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MICROBIC DISSOCIATION

THE INSTABILITY OF BACTERIAL SPECIES WITH SPECIAL REFERENCE TO ACTIVE DISSOCIATION AND TRANSMISSIBLE AUTOLYSIS

SIX PLATES

Philip Hadley

From the Hygienic Laboratory of the University of Michigan, Ann Arbor

OUTLINE

1. INTRODUCTION

For the past three decades there has been accumulating an ever increasing mass of evidence pointing to the instability of bacterial species. For the systematic bacteriologist the situation has already become somewhat alarming; while, for those who have never taken the problems of classification too seriously, it at least has inconvenient aspects. But the present state of affairs, as reflected in both publications and discussions, may not be entirely free from elements of danger, since the growing chaos tends to discourage sincere attempts to alleviate the difficulties by searching for the causes that underlie them. While it may be true that greater knowledge of systematic relationships may not contribute significantly to useful knowledge of the bacteria in this or that relation, the inherent problem involved concerns closely microbic variation; and this, in turn, may have a more significant bearing on problems of virulence, infection and immunity than many have supposed.

When confronted on every hand with such pictures of bacterial instability, certainly of a pattern too extensive and intricate to admit of clear exposition, but the general nature of which most bacteriologists realize, it is logical, first to inquire whether the confusion we observe is pure chaos, or whether there exists any trace of orderliness amidst the general disorder, the extreme possibilities of which were first pointed out by Nägeli.[361] Cocci become rods and rods cocci or spirals; forms of growth change overnight; motility is lost and regained; fermentation reactions are modified by time and opportunity; spore formers become sporeless; hemolytic activity comes and goes; capsulated bacteria lose their capsules, and capsules are gained by noncapsulated forms; antigenic power vanishes and reappears; cultures become spontaneously agglutinative or fail of agglutination; virulent cultures become harmless and harmless cultures virulent. It may be many years before we learn

the cause of all these apparent incongruities; but we are bound to attack
the problem as best we may and to grasp at any possible thread of
orderly change running through the tangled web of bacterial variation.

I believe that sporadic citations in the literature of the past thirty
years, and more frequent references within the past ten years, indicate
that there exists such a thread, common to at least some of the changing
bacterial patterns.   Indeed, it is becoming clear that one possible
approach to the problem may be established through the medium of a
new but definite branch of bacteriological study which is just beginning
to emerge from the state of scattered observation, and to assume organ-
ized form.   It is one, moreover, which has attracted less attention than
it deserves; and which, it may be predicted, will soon be found to
possess unlooked-for significance in its bearing on several important
problems relating to bacterial variation and "mutation," serologic reac-
tions, virulence, immunology and serum therapy.   I refer particularly
to a study of certain more or less orderly mutation-like changes in
bacterial cultures, often marked by autolytic processes and occurring
either more or less spontaneously, or under the influence of the bac-
teriophage or lytic principle.   Certain aspects of this new study thus
concern the phenomenon which has been termed "microbic dissociation,"
while others are related to transmissible bacterial autolysis.   Still others
are more clearly related to culture and cell changes that have been
reported by a small group of investigators in connection with the
so-called "life cycles of bacteria."   The chief point, however, is that
there has been slowly developing in bacteriological literature a large body
of scattered, and up to the present time uncorrelated, data regarding
peculiar culture changes and species instability which, although little
appreciated by the majority of bacteriologists and lacking adequate
explanation and interpretation, promises to serve as an entering wedge
into some of the inscrutable problems that today confront us.   And it
may be prophesied that, when more of the facts are known, it may be
shown that many aspects of observed bacterial instability, far from
being a sign of chaos, are in reality indicative of the action of certain
biologic laws which are orderly in their operation and widespread in
their application among unicellular microorganisms.

It is the chief aim of this paper: to expand this view regarding
microbic dissociation; to examine in some detail its causes, modes of
expression and effects; to make certain comparisons between microbic
dissociation and transmissible autolysis with special reference to the
mode of action and effects; to suggest forms of reference for some of

the apparently more important "types" of bacterial cultures involved—
by means of which thinking may be kept clearer and the interchange
of ideas made more effective; to reexamine the nature of bacterial
"species" and of bacterial "mutations" in the light of the dissociative
process; and to consider the probable biologic significance of dissocia-
tion in the life of the bacterial culture and of the bacterial species.

To all this it may be added that this work has one further purpose
which, though not announced in every section, it is hoped will be kept
in the mind of the reader throughout. It relates to the problem of the
bacteriophage (transmissible bacterial autolysis), about which a further
word may be said at this point.* In the field of bacterial autolysis, as
we view it today, there are two classes of phenomena demanding special
attention. The first of these is microbic dissociation; the second is the
classical phenomenon of d'Herelle, termed by him "bacteriophagy," but
by Bordet [62] "transmissible bacterial autolysis." Regarding the first,
which has received little attention up to the recent time, there is small
question regarding its normal, and apparently spontaneous, nature,
although the reaction may easily be forced. Regarding the latter, how-
ever, there exists a division of opinion regarding the causal agency—
one school (that of d'Herelle) maintaining the bacteriophage (Protobios
bacteriophagus, syn. Bacteriophagum intestinale d'Herelle, 1918) to be
of a living, virus-like nature; the other school (that of Bordet) main-
taining the phenomenon to be due to an inherited, vitiated metabolic
state of the organism concerned. Whatever may be the relative merits
of these respective views, it is perhaps of importance to note that both
bacteriophagic action and microbic dissociation (as this subject will be
developed in the following pages) have a common meeting ground in
a single aspect of physiologic behavior—namely the instability of the
bacterial culture in its reproductive function. In view of this fact it
has seemed worth while to conduct a detailed study of phenomena
bearing on this subject so far as rendered possible by the literature;
and this with a view to ascertaining to what extent, if at all, we can
detect any relation in cause, nature or mode of expression, obtaining
between these apparently divergent phenomena, proceeding out from a
presumably common physiologic state. I believe it is only by a study of
such a comprehensive sort that we can derive a sound support for logical
and effective methods of investigating the nature and meaning of bac-

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* If the reader is not already familiar with the phenomenon of the bacteriophage (the
d'Herelle phenomenon), much that appertains to this phase of the subject will be to him mean-
ingless. The reading of d'Herelle's most recent (1926) and fully comprehensive work [248] is
recommended. For an understanding of the most widely recognized alternative view relating
to the theory of the bacteriophage, I would recommend a fairly recent paper by Bordet.[61]

teriophagic action, about which, notwithstanding the most recent and
elaborate researches of d'Herelle and his supporters, one must believe
the last word has not yet been said.

2. BRIEF CHRONOLOGICAL REVIEW OF THE DEVELOPMENT OF OUR KNOWLEDGE REGARDING DISSOCIATIVE PHENOMENA

In surveying the present state of our knowledge of microbic insta-
bility, and particularly that phase of it which has become known as
microbic dissociation, as this will be presented in detail in later pages
of this review, it will prove most convenient to regard it as related to
certain special fields of bacteriological thought and practice. In order,
however, to present a clear notion of the course of development of our
knowledge in these fields, it may be of advantage to preface this con-
sideration with a brief résumé by means of which there may be brought
to light a few of the more important events in their chronological order.

Our present knowledge of microbic dissociation has its roots in
numerous older observations on microbic variability and assumed trans-
mutations. The early and common notions of transmutability among
the fission fungi, current through the middle of the nineteenth century,
were crystallized in the views of Nägeli,[361] published in his Lehrbuch of
1877. For him the fission fungi represented only a single type of cell,
highly variable and capable of passing from one morphological state to
another, as well as capable of undergoing profound alterations in bio-
chemical behavior and fermentative capacity. Thus, a "generic and
specific" differentiation of the bacteria on the strength of their morpho-
logical and biochemical characters was not justified. By this time,
however, the botanist, Cohn,[103] had begun to insist with equal vehemence
on the conservatism of bacterial types and had presented a system of
genera and species which was based solely upon morphological and bio-
chemical grounds. This conception of constancy of species formed a
part of the inheritance of Robert Koch, whose discoveries
and observations, together with those of his disciples, succeeded in
forcing upon the bacteriological world a saner—perchance too sane—
view definitely opposed to that of Nägeli and his colleagues—namely, a
conviction of the constancy of bacterial types. In bacteriological thought
the pendulum of opinion regarding the fixity of species, in these early
days, was swinging wide, and the Cohn-Koch views gradually became
established as a sort of dogma, in that there was recognized for each
bacterial species only one limited colony and culture type, all the cells
of which were characterized by the same predetermined morphology.

As Baerthlein [30] pointed out years later, there arose in this way conceptions of normal colonies and normal bacterial morphology, which have never ceased to influence—and in later years, unfavorably—both bacteriological thought and practice. What did not conform to the "normal" was relegated to the field either of degeneration or of "involution" forms—a mischance in which in later years Loeffler and others of his school did much to assist. These degeneration and involution forms have served, even to the present day, as the final repository for all forms of all bacteria which depart from expected "normality."

Although, during the eighties and after, the trend of bacteriological thought turned strongly to the side of Koch's laboratory and here found strong support in the monomorphic views of Migula [353] (1897), other writers, notably Hueppe, Kruse, and Gruber, held tenaciously to the conception of bacterial variability, although perhaps not to the extreme view earlier held by Nägeli. The persistence of these views found expression particularly in the characteristic work of K. B. Lehmann and Neumann, [301] the "Systematik und Handatlas," familiar to all students of bacteriology. In the micrococcus group, in Bact. pneumoniae, Bact. acidi-lactici, B. lactis-sporogenes and in the pyocyaneus-fluorescens group, they pointed out that many accepted species were often only the expression of variability in a single species. These as well as succeeding intimations of bacterial variability, such as those of Neumann for M. aureus, of Kossel and Kolle and of Kruse for the vibrio of cholera ("Stamm VI"), of Gruber and Firtsch for the vibrio of Finkler-Prior, of Schottelius and Dieudonné for B. prodigiosus—all fell on deaf ears. The results obtained were commonly regarded as due to contamination; or, if they were accepted as facts, it was believed that they possessed nothing of practical significance. The dogma of the absolute constancy of specific bacterial types was strongly entrenched.

It probably can be said with truth that slight general interest in, or acceptance of, the fact of bacterial variability even in a relatively minor degree (to say nothing of the "transmutability of species" as upheld in extreme form by Nägeli, the Buchner brothers and others of their school), was apparent until about 1906 when Neisser (1906), and Massini (1907) injected into the somewhat torpid literature of the day their observations on "mutations" in a strain of B. coli, termed B. coli mutabile—observations too clearly significant to be brushed aside. Although at once registered as contaminations by Kolle, they were fortunately quickly confirmed (at Kolle's instigation) by Kowalenko

(1910) who took the precaution to work with single cell cultures. These results, apparently remarkable enough at the time, were quickly extended into other fields. The result was to bring into the field of study of bacterial variation the de Vriesian term, "mutation," imported from the botanical literature. The work of Neisser and of Massini served as a stimulus to many similar observations and the literature from 1906 to 1914, especially in the English school, is rich in allusions to mutation-like phenomena among the bacteria. Many of these observations, particularly from German sources, are summed up (to 1914) in the splendid monographs of Eisenberg. Eisenberg attempted to analyze the various types of variation and to classify them according to their significance—modifications, fluctuations and permanent changes, the last referred to by the terms, mutation, clone-formation, etc.

Regarding the justification of the terms, modification or mutation, much has been written in subsequent times, but without leading to fruitful results. Some investigators have observed cultural alterations in countless numbers believed to be of mutational significance, while others, as Ernst Lehmann, have regarded such variations as observed by Neisser and Massini as not entitled to the term, mutation, which according to their view should be reserved for organisms possessing a sexual form of reproduction. Lehmann held the same view for the "pure lines" or "biotypes" of Johannesen. As we shall see later the question of the justification of the use of the term, "mutation," depends entirely upon the significance which we shall attribute to the phenomenon of microbic dissociation as the subject will be evolved in the following pages.

But perhaps the greatest significance attaching to the controversy over mutations lies in the circumstance that many bacteriologists were admitting the existence of some sort of bacterial variation; were, indeed, recognizing wide, and sometimes apparently permanent, departures from the long assumed, constant "normal type."

The expression of these views, from the time of Neisser's and Massini's work on B. coli mutabile in 1906 and 1907, up to about 1921, which marks the beginning of another stage in the study of microbic instability, took various forms. Indeed, it is seldom that we see the problem attacked consciously and directly. Practically all the earlier observations of present significance are byproducts of some other work; and in many instances were not recognized even by the authors themselves as being related to the general problem of mutation. Some of the observations were concerned with morphology or cultural type, some

with biochemical reactions, some with serological reactions and others with virulence. These aspects of the general problem will be considered independently in due time. But it is of interest to point out in passing that, subsequent to the first observations on the agglutination phenomenon by Grubler and Durham in 1896, many studies on the discrepancies in this reaction played indirectly into the hands of the "mutation problem." These studies began with the work of Nicolle (1898) on self-agglutinating cultures of the typhoid bacillus. They were carried forward rapidly by the English school, to a considerable extent by Savage and his followers, and culminated in the valuable work of Arkwright in 1921 dealing with dissociation as a recognized phenomenon among various intestinal bacteria.

Extending more or less parallel with this study of variation in its relation to serologic reactions was a study of the variants in relation to fermentation power. These studies were initiated soon after the introduction of B. coli mutabile by Neisser (1906) and were prosecuted particularly in England by Ledingham, Penfold and their associates as well as by Reiner Müller and others in Germany. They culminated in the significant work of Müller and of Penfold on daughter colony mutants in members of the colon-typhoid-dysentery group. In Germany the study of variation was carried further by Eisenberg in his splendid series of papers on bacterial variability; also by Bernhardt who in 1915 introduced several new and significant points bearing upon the dissociation problem as we have come to know it today. In 1917 appeared the work of Weil and Felix on B. proteus X19, in which unknowingly they approached the dissociative reaction from a different viewpoint; but in such a manner as to make their results converge with other data towards the central problem.

Although these early studies were largely concerned with variability related to serologic and fermentative reactions, the field of morphological variation in colonies and cultures, as a point in itself, had not been abandoned. In 1918 appeared the classical work of Baerthlein representing a study of colony variation and correlated features in fourteen common bacterial species, each analyzed with a wealth of detail. This important work not only correlated many previous observations regarding purely morphological aspects of colonial and cultural variation, but extended the observations in such a manner as should have cleared away, once and for all, any doubt regarding the absurdity of the old dogma of "normal bacterial types"—at least in the extreme form in which it was maintained during the Cohn-Koch regime. At the same

time, any far reaching conception of variability that could have been based on Baerthlein's observations must have fallen far short of the extremes of bacterial transmutability pictured by Nägeli and others of his school.

While we may well hesitate in attaching significance to all of the various colony types of each organism described by Baerthlein as actual variations or mutations, there can be no doubt that, among these types, there exist two or three significant forms which we shall see play an important part in microbic instability and dissociation. The most important fact clearly established by Baerthlein's exhaustive and detailed treatment is that colony-variability may always serve as a criterion of cultural variability; moreover, that with the various colony types are closely correlated other highly important characteristics of the organism, including individual morphology of the cells, pigment production, slime production, fermentation reactions, serologic reactions, and virulence. With reference to these matters, Baerthlein appears to have been the first to grasp in a broad way a view of some of the important consequences of bacterial instability; and to place on record a mass of valuable data much of which we are able today easily to interpret into the phenomena of microbic dissociation.

Probably the first investigator to recognize the fundamental significance of Baerthlein's contribution to the study of bacterial variation was Arkwright, already mentioned, whose studies possessed the merit of getting at the kernel of truth which, in Baerthlein's study, was somewhat overshadowed by a wealth of detail often including some inconsequential aspects of variation. Arkwright apparently recognized among the many colony variations reported by Baerthlein two which were of special significance; and these he was able to identify in the case of many organisms of the colon-typhoid-dysentery group. They are the socalled rough (R) and smooth (S) types. He pointed out clearly the trend of the dissociative process (which had already been observed by Baerthlein) from S to R, but not commonly nor easily from R to S. He pointed out again the characteristics associated with the R and S types, their cultural and biochemical features and their serologic inter-reactions; and discussed the significance of these variations from the viewpoint of the mutation hypothesis.

Although the fundamental work of Baerthlein attracted but little notice at the time of its publication, the significance of Arkwright's contribution was seized upon at once, first and foremost by the English school, while in this country the same line of experiment was developed

independently in the hands of de Kruif. In England Arkwright's observations were at once carried over to the streptococci by Cowan (1922), to the pneumococcus by Griffith (1923), to B. enteritidis by Topley and Ayrton (1924) and by Goyle (1926); also to Salmonella types by P. B. White in 1925. They also underlie the chief significance of the dissociative features of the studies by Atkin on the meningococcus (1923) and on the gonococcus (1926). They were carried over to the work of Arkwright and Goyle in 1924 on a comparison of the S and R strains of B. typhosus and B. dysenteriae with the H and O forms of Weil and Felix, and continued in this direction by Goyle in a report of exceptional value appearing in 1926; also by Balteanu in his thorough antigenic analysis of the cholera vibrio in 1926.

In 1921 de Kruif also had reported on the dissociation of Bact. lepisepticum of rabbit septicemia, clearly demonstrating the S and R types, the former virulent, the latter not. De Kruif's results were extended into interesting fields by Webster. The work of Griffith (1923) on the pneumococcus was confirmed and extended by the studies of Reimann and of Amoss in 1925. All of these last studies have served to confirm in a somewhat startling manner the relation of the dissociative reaction to the problem of virulence as well as to many other peculiar aspects of bacterial behavior.

Suffice it to say in concluding this brief chronological review of the earlier studies bearing directly or indirectly upon the dissociation phenomenon, that the data now accumulating, although not meeting by any means Nägeli's surmise regarding the extent of transmutability of bacterial species, can unquestionably be accepted as indicating the existence of a highly unstable state of the average bacterial culture when placed under conditions of changing environment; and as indicating likewise a common trend in the variational processes in all bacterial species so far observed.

As we proceed with the following sections of this review we shall find our inquiry leading into many fields of bacteriological thought and practice. And as we do so we may remind ourselves of a phrase from a work of Laurent.[297] Referring to the old question of bacterial variation he said: "Assurément le transformisme indéfini des Bactéries rèvé par l'ancienne école polymorphiste n'est qu'une chimère. Mais quel vaste champ de recherches reste à explorer dans la voie des variations physiologiques des microbes!"

So little has the science of bacteriology progressed in the past three or four decades, so far as our intimate knowledge of the bacteria is

concerned, that these words, uttered with reference to the old "Ruber of Kiel," come to us today almost as freshly significant as when they were written more than thirty-six years ago.

3. THE NATURE OF THE REACTIONS INVOLVED IN MICROBIC DISSOCIATION

In the earlier development of bacteriology, as has been shown, the predominating conceptions of the constancy of specific bacterial types, taken in conjunction with the fear of unwarranted generalizations, has undoubtedly done much to perpetuate the view that each species was a "law unto itself" with respect to its kind of variation and physiologic behavior. But we are now beginning to see that, as in microbic respiration for example, as this subject has been illuminated by Novy and Soule,[375] certain fundamental aspects of physiologic behavior are not peculiar to this or that species, but are the property of many, and probably all, bacterial forms; and perhaps of all living protoplasm. In a somewhat similar way it is becoming clear that there may also exist fundamental trends of variation in bacteria; and that these are observable, not only in different genera, but also in different families and orders, and probably in higher forms such as Cladothrix, Ascomycetes and the higher fungi as well.

In the majority of scattered instances in which significant dissociations have been noted the study of the variations themselves, as I have already pointed out, has seldom been the primary aim of the inquiry. They have been noted "in passing," so to speak; and our present knowledge of them has been built up largely through resort to reports on widely different lines of research, as for example: the effect of dyes on intestinal bacteria, or of antiseptic substances on typhoid or anthrax bacteria; the "spontaneous agglutination phenomenon," variations in virulence of many species, varying reactions to phagocytosis, the "double antigen" problem of Proteus X19, the body and flagellar antigen of Smith and Reagh,[445] secondary colony formation, and various other bacterial adaptations to unfavorable environments. In other instances they have involved merely casual observations on colony form or other physical aspects of growth. Moreover, it is seldom, at least up to recent times, that an investigator has seen, in his own results, a phenomenon analogous to other and similar phenomena described earlier or elsewhere. Few attempts have been made to study dissociation in itself; and, so far as I am aware, no attempt has been made to correlate these interesting observations or to indicate the extent to which many of them contribute

to the formulation of a general scheme of variation and adaptation underlying bacterial instability. First, however, we must understand the chief features of the dissociative process as it occurs on solid and in liquid culture mediums. The observations immediately following have been made by so many laboratory bacteriologists that it is unnecessary, except in more exceptional cases, to cite original references. I wish to present here merely a general picture of dissociation phenomena as they occur on common culture mediums and with ordinary cultures such as B. coli, B. typhosus, Friedländer's pneumobacillus, B. anthracis, B. subtilis, B. pyocyaneus, etc. Next we shall turn to some less obvious and more unfamiliar instances in which it is apparent that dissociative reactions also play the predominant rôle, especially in biochemical and serological reactions.

Dissociative Phenomena on Solid Culture Medium.—Superficially at least, microbic dissociation involves the partial or total transformation of a pure line culture of normal type into one or more subtypes often differing in cultural, morphological, serological and biochemical characters from the original. The phenomenon may be accompanied by the slow or rapid disappearance of the mother culture. If it occurs slowly no macroscopic culture changes simulating lysis appear; if rapidly, a sort of disintegration or lysis of the old culture mass may be seen to occur over broad or limited areas.

Dissociation may occur naturally (spontaneously) or it may be forced by appropriate measures. It may occur in colonies or in broad surface growths on solid medium, or in liquid medium. Naturally that occurring in colonies is the most significant if we are led to believe that the colony has been founded by a single cell. Much of the later work involving dissociation (de Kruif,[118, 119] Webster,[481] Reimann,[405] Amoss,[8] Mellon,[325, 344] Jordan,[273, 274] has involved this precaution. These results, however, are in no way different from the earlier results involving colony isolation only. It is highly important that certain recent work involving single cell isolations seems to validate many earlier observations in which only colony isolations were employed; and even some in which apparently only mass inoculations were practiced, as in the early but important observations of Bordet and Sleeswyk[64] on B. pertussis in 1910.

The phenomenon reveals itself on solid culture medium in many ways. These include primarily: secondary colony formation without erosive action; secondary colony formation with erosive action; lysis and transformation over limited or broad areas without

erosive features and with delayed secondary colony formation. These
reactions, involving a greater or less disappearance of the old, normal
culture mass and the generation of new bacterial forms, may occur
either in mass cultivations on agar or in single colonies.

Formation of Secondary Colonies: In the simplest and probably
most commonly observed cases dissociation occurs in the form of
colonies within colonies without any striking signs of lysis. The old
colony, having attained fair growth, undergoes a sort of degeneration,
becoming glassy and translucent, not in patches, but over the entire
surface. Within it arise daughter colonies or papillae (Knöpfe), from
two or three to 100 or more, depending on the colony size. When such a
colony, especially if old, is cultured in mass it is often only the daughter
colonies that yield growth, although the culture in the glassy areas may
sometimes perpetuate itself, if not too old. If the subculture is made
early in the life of the primary colony both types of organisms will
appear in varying numbers. Sometimes the old culture disappears only
slowly and well formed papillae are present after three to seven days.

Although Günther [218] had seen and described secondary or daughter
colonies as early as 1895, Preisz [393] (1904) was one of the first to
mention typical and atypical strains of B. anthracis in relation to forma-
tion of secondary colonies (Knötchen) and spore formation. He made
use of slightly alkaline agar and on such medium showed how variations
in the form of the rods went hand in hand with cultural variations. The
colonies observed when one day old might be either whitish or bluish,
smooth or rough, sharply delimited or indistinct, rich in spore produc-
tion or weak. These characteristics usually appeared divided between
the two chief types of colony in the relations mentioned, the former
perhaps representing the S type, the latter the R. In some of the larger
and more translucent colonies there arose, sometimes after a day or
two, sometimes only after a week or more, granular, half-moon shaped
"Knötchen" which slowly transformed themselves into "secondary
colonies." The translucent races which formed spores most quickly
and richly always gave primary colonies with the most numerous sec-
ondaries. After a time the centers of these secondaries became pale
and translucent, sometimes star-shaped and surrounded with a slightly
raised, whitish ring, as if the transparent center had been drawn back
to the periphery. Sometimes similar clear areas arose in the primary
culture mass itself, without the presence of obvious secondary colonies,
a point of extreme significance as we shall see later. The secondaries
sometimes showed a "ring-structure," with the clear center still intact.

Preisz attributed the formation of the secondary colonies to the germination and subsequent growth of spores, forming a new race of young bacilli of quite different form from that of the original colony. The consistency of these colonies was often tough and tenacious, so that the entire colony could sometimes be removed from the medium. This phenomenon is very characteristic of many "extreme" R forms. As sporelessness increased, the primary colonies became bluer and the rods longer and more filamentous. Such races, Preisz held, were "headed for destruction"; the cultures would "run out." In conclusion, he noted similar secondary colonies in colonies of B. diphtheriae, Vib. cholerae and Sp. Finkler-Prior. The secondary strains always possessed the greatest longevity. In closing he makes the following pertinent (in relation to our problem of dissociation) observation: That asporo-genous as well as sporogenous bacterial species can show exceptional individuals whose vital power is attributable to a splitting of the race; and that it is perhaps by reason of this diversity of type that many statements regarding tenacity of life of nonsporeformers are so little in accord.

The phenomenon observed by Preisz involved important aspects of dissociation and is of unusual interest, not only for its bearing upon variation, but also because of its apparent relation to bacteriophagic phenomena. In this aspect it has been treated further by Pesch and by Katzu whose work will be reviewed shortly. Preisz showed: first the origin of secondary colonies within primary colonies of the blue trans-lucent R type; second, the lysis of the centers of these colonies, with the production of erosions; and third, the occasional appearance of tertiary colonies on the sites of lysis. Here, it may be noted in passing, we have in a rough way, appearances suggesting the production of the lytic areas by the bacteriophage. The only striking difference is that, although Preisz could clearly recognize the source and origin of his lytic spots (Löcher), we do not know the origin of lytic areas. Both Pesch and Katzu noted the resemblance but were unable to detect the presence of a filtrable lytic agent.

The formation of tertiary colonies, as reported by Preisz is of special interest. These were observed to form in the bare areas pro-duced by the lysis of the larger secondaries, and were attributed to the germination of still other spores remaining after the dissolution of the first spore colony lying within the primary. The same sort of phenomenon was reported later for B. anthracis by Pesch,[387] who extended the original results by the following observation. The smaller,

whiter and more compact anthrax colonies after some days sent out
from their borders a thin, transparent and expansive growth simulating
the structure of the blue or translucent colonies. If, however, the
plates were held longer at 37 C. there arose colonies of the opaque,
whitish type in the blue, marginal growth. Seeding from these gave
a mixture of both colony types. He also noted that the blue or trans-
lucent form of growth appeared to "repress" the growth of the whitish,
opaque type. As suggested later, we may provisionally regard the
whitish colony as the S form and the translucent as the R.

Daughter colonies were later observed in many bacterial species:
In the intestinal group by Neisser,[362] Massini,[320] Kowalenko,[286] Burri,[88]
R. Müller,[356] Thaysen,[460] Penfold,[385] Baerthlein [27-30] and many others,
including Eisenberg [148] who in 1906 described daughter colonies in a
considerable number of species. Similar observations were made by
Beijerinck [42] for B. nitroxus and by Engelland (according to Enderlein)
for M. albus and M. aureus growing on agar containing tartar emetic.
Here the secondaries, which were much larger and more strongly
colored, overgrew the smaller primary colonies. Atkin [21,22] has recently
studied daughter colonies of Diplococcus gonorrheae and the meningo-
coccus. Enderlein [159] reported brilliant red daughter colonies of
B. prodigiosus in colorless primary colonies grown on agar containing
from 6.5 to 8% of salt. It is undoubtedly true, as Enderlein [160] states,
that daughter colonies may be observed at times in cultures of all species
of bacteria. In some they may appear within a few days, while in
other species they may not be seen until the cultures have grown for
several weeks, or even months.

On the other hand, as Enderlein also notes, it may sometimes be
difficult or even impossible to ascertain whether a primary colony pro-
duces daughter colonies. This can result either from the circumstance
that they may be so small as to escape notice, or that they
may be so numerous and develop so quickly, that they fully obscure
the primary growth. Often minute areas of granulation represent the
beginning of secondary growth; and these may occur either deep in the
colony mass, superficially, on the surface, or at the rim. All colonies
should naturally be examined with a hand lens as well as with the
No. 3 objective. If a colony contains only one type of organism (i. e.,
that of the mother culture itself) it is, according to Enderlein, an
isomorphic colony; while if it contains two or more types of culture
(cyclostages), it is a heteromorphic colony. Aside from the statements
of Enderlein, and the older records of Preisz [393] (1904) regarding

tertiary colonies in the case of B. anthracis, I am not aware that more than one kind of secondary colony in a single mother colony has been reported. This phenomenon has, however, been confirmed with respect to giant colonies of Streptococcus fecalis growing on Gordon deep "trypagar" plates by Faith Hadley.[499]

We may now ask ourselves—what is the nature of the daughter colonies arising as secondaries in the mother colony? One might be inclined to consider them as representing the R culture formed within the S form. This may be the case in certain instances, and particularly in those cultures in which secondary colonies form in the culture background of old and desiccated agar slants; but there are certainly exceptions; and these are found chiefly in colonies having an age of three to seven days and observed before the effects of desiccation can have become prominent. Under such circumstances, the chief difficulty in the way of interpreting the daughter colony "mutants" as "rough" forms is that, neither in the papillar state nor in subcultivations from papillae, are the centers of modified growth rough. Indeed, they are much more likely to be smooth, as anyone can ascertain by simple examination with a hand lens in good reflected light. This fact is shown particularly in daughter colonies of Streptococcus fecalis, Streptococcus mitis, and Streptococcus salivarius (Faith Hadley [499]). Moreover, Reiner Müller [357] has indicated that when one subcultures a papilla occurring in a typhoid colony the resultant colony growth is often a large, mucoid colony which, in turn, sends out after some days a delicate film (regeneration fringe) representing the normal culture form. These mucoid colonies therefore appear to be "intermediate" and can regenerate the original culture. They are, however, very unlike the R type colonies. Often, as shown by Krumwiede and his collaborators,[288] the mucoid form (B. paratyphosus) may generate the true R culture, rather than the normal S. The same point has been shown by several others for other bacterial species and will be considered in detail in section 6 of this paper. That the true R type colony may, however, develop in the mother culture mass after many days or weeks has been shown for Streptococcus fecalis by Faith Hadley.[499] Here, in giant colonies measuring from 12 to 18 mm. in diameter (on Gordon's trypagar plates grown for several weeks) daughter colonies accompanied by distinct papillae made their appearance in a few days. It appeared that, if the secondary colonies occurred near the surface of the mother colony, they would form papillae; if the colonies were deeper no papillae resulted. The first colonies (and

papillae) to arise were coarsely granular by transmitted light, but at the same time smooth by reflected light. After two to three weeks, however, a second type of daughter colony might arise, lying much deeper and sometimes imbedded in the agar itself. These were extremely rhizoid and gave no resemblance to a streptococcus colony. They might be easily mistaken for crystalline deposits unless one removed such a colony in toto, crushed it on a slide and examined stained preparations. Here there appeared a streptococcus in long chains, quite different from the typical fecalis which shows only a slight tendency to chain formation. When transplanted to fresh medium such colonies produced a certain number of "extreme" rough colonies having a thin, coarse, dry and coarsely granular structure and an irregular, jagged margin with many filaments extending out into the medium. Among these roughs were several other very peculiar colony forms. When, on the other hand, the smooth secondary colonies were transplanted to fresh medium many reverted to the normal type, while others began to take on R characteristics and with further repeated transfer to approach more closely the true R type. Streptococcus fecalis colonies were thus found to give rise to at least two forms of daughter colonies, one arising early, the other appearing only after a longer interval and typifying the R form. The subject of daughter colonies in this organism is, however, very complex and requires much further study.

Regarding the general question of daughter colonies a few other points may be mentioned. Regarding their location in the colony, they may appear either in the central part or at the rim. There may be only a single one, or there may be so many that they quickly become confluent and give such an appearance as to suggest that the whole colony is made up of the daughter colony type of culture. If they appear only at the rim of fresh growth, they present the appearance of a rosette. If, in such a structure, the colonies fuse together they produce the effect of a rim-wall about the colony which then manifests a depressed central area. If the original culture regenerates circumferentially outside the rim, then after a time another rim of secondaries may be formed, thus giving concentric zones of growth such as have been often observed by bacteriologists. Enderlein [160] described in detail some of these colony pictures, and they have been reproduced in photographic form by Eisenberg [148] in his interesting study of secondary colonies in 1906.

In the cases mentioned in the preceding pages dissociation usually occurs without striking signs of lysis in the primary colony mass other

than a gradual "melting away," and increasing transparency, of the old culture mass, such as is often seen to accompany the process of aging. There exist, however, two other types of dissociation characterized by greater speed of reaction ("suicide cultures"), and sometimes by marked erosive disturbances in the colony or culture mass. The suicide culture has no doubt often been seen by bacteriologists though seldom described. Collins [106] has reported a culture of this sort from a rabbit abscess. I have studied one coming from the gastric mucosa of a rabbit dying in course of immunization to B. typhosus; also from air of the laboratory. Probably it was such a culture of slow dissociative reaction described by Moto [359] as his "creeping culture" (B. helicoides). These cultures are characterized by extremely rapid transformation and often leave in their wake a secondary growth. This type will receive further consideration on a later page.

Erosive Phenomena: Dissociative reactions characterized by greater speed and observable either in colonies or in broad surface growths also occur in the form of erosive phenomena. This was first described for colonies of B. anthracis by Preisz [393] in 1904, and has been rediscovered at intervals since that date. It also appears in the dissociation of B. pyocyaneus as this has been described by Canzik [89] and others including myself.[224] The same phenomenon is undoubtedly concerned in the peculiar erosive ("bacteriophageähnliche") reactions seen by Sonnenschien [448] in a Monilia culture from the throat; in B. cereus (Andervont and Simon [9]) ; also in cultures of Saccharomyces.

The phenomenon of dissociation in B. pyocaneous shows strong resemblance to the Preisz phenomenon in B. anthracis; but with this exception: It has been impossible to determine the origin of the erosions. They appear to develop from areas of granulation, as in the Preisz phenomenon, but it has not been demonstrated that they develop from colonies, although this seems highly probable. In the case of pyocyaneus the granulations which subsequently may be centers of erosive action make their appearance first on the surface of the colonies (on agar slant cultures) as metallic flecks. These gradually enlarge and deepen to produce erosions of large size and with perpendicular walls. Over the floor of the pockets, as was also true in B. anthrax, lies a thin film of culture.

The phenomenon observed and pictured by Sonnenschien in Monilia shows striking resemblance to the case of pyocyaneus. Unfortunately, however, he made no reference to different types of colony or variations

in cell form.   He found no evidence of transmissible lytic action such as
I have observed in pyocyaneus.

Transformation and Delayed Formation of Secondary Colonies:
Still another manifestation of the dissociative reaction involves the
appearance of a fringe of new and modified growth at the border of a
colony or streak, from which it extends in a delicate film like a halo
(regeneration fringe).   It was pictured by Sanarelli [423] in 1897 for the
cultures of B. icteroides obtained in his yellow fever researches.   It is
probably the same reaction as the "fringe growth" on colonies of
B. anthracis described by Pesch, as already mentioned.   It has been
described moreover by Neisser and by Massini for B. coli mutabile
and can be seen in almost any plating of sewage polluted water on Endo
plates, as I have often observed when they are kept for a week or more
at room temperature.   It has also been reported by Bernhardt,[49]
Baerthlein [30] and others.   I have observed the phenomenon in colonies
of B. diphtheriae, B. malleus, staphylococcus and in a proteus-like
culture.   Possibly a similar growth has been mentioned by Braun and
Schaeffer [66] for the O form of proteus X19 of Weil and Felix, which
is described later.   It does not ordinarily issue from a normal culture,
but from an intermediate form (see section 6).

Dissociative reactions may show themselves also in another manner.
In cultures of many intestinal bacteria, Friedländer's pneumobacillus,
etc., grown upon alkaline (P_H 7.8 to 8.0) agar the borders often show
curious bluish or translucent, wedge shaped invaginations which may
unite to produce a slowly growing translucent fringe.   The lysis of
staphylococcus colonies observed by Twort [468] was probably a closely
related phenomenon.   This has been mentioned by Gratia for B. coli,
and I have seen it in nearly all members of the intestinal group.   It
likewise may appear as blue sectors in colonies, when they are from a
few days to a week old.   The translucent fringes and invaginations in
such cases are made up almost exclusively of R type culture, while the
S form has largely disappeared from these areas.   If such areas are
plated they yield almost exclusively thin, irregular, translucent colonies.
It is important to note that in cases of this sort, there exists not only
an extension of the fresh growths to new agar surface, but also an
extension of the dissociation area backward and into the original culture
mass which may eventually be wholly consumed, leaving only a thin
film of transparent growth (Hadley [226]).   This reaction I have
described and pictured in another place for some unknown air and
water bacteria.   The colonies may become so transparent as to make

easily possible the reading of printed type lying beneath the plate. Such cultures are in reality, "slow suicides." They have been clearly pictured, but not adequately described, by Gildemeister [195] in 1916 for a culture of B. paratyphosus B, isolated from a carrier.

In connection with growth fringes, regeneration fringes, or "halos," it is important to observe that cultures of the R or of the intermediate type (O) may arise (though indirectly, as we shall see) from the S; or that cultures of the S type may arise (perhaps directly) from the R. The former is certainly the more common. The growth fringes of B. proteus, as described particularly by Braun and Schaeffer,[66] are of interest in this respect. Here, however, the pure R type is perhaps not yet definitely known, but we have two clearly marked forms, the spreading (H) and the restricted (O) to be considered later in detail. In this case the intermediate O sends out the fringe of H. In proteus cultures many bacteriologists of laboratory experience have observed the "ring-growth," in which, starting from a single, small colony on a plate, eight to ten or more concentric rings of alternating transparent and whitish growth may be found spreading from the common center. I have studied several of these cases and have concluded that they represent alternations of S and O culture indicating alternate dissociations and recoveries. The true R form, however, was not present in the cultures which I have examined. The characteristic S or O cultures can be isolated from the respective ring areas. It seems likely that "ring-growth" in some other bacterial species may have a similar explanation.

Certain observations, however, particularly on B. proteus, make it appear that the question of regeneration fringes may not always be so simple as the regeneration of typical R (or O) from S or of typical S from R culture. The point in question has been made clear particularly in a study by Braun and Schaeffer on the double antigenic proteus HX19 and the single antigenic OX19, as well as on X2 strains. In view of some later deductions it is important to present this matter in some detail.

Braun and Schaeffer attempted to ascertain whether the HX19 of Weil and Felix [483] could be transformed into OX19 by disinfectants or by "starvation" (for methods see section 11). By growing on phenol agar and on agar impoverished in beef-tea content they succeeded in obtaining the O type which was fairly constant in its changed (non-spreading) character in contrast to the H type (spreading), even when returned to common mediums. It sometimes happened, however, that

these O type cultures (and particularly a natural OX19 obtained from Weil) gave a regenerated growth quite different from the original H though possessing the H spreading character. This new growth, which emanated from a single streak of OH19 on an agar plate, eventually spread over the entire plate. Unlike the normal HX19 (also spreading), however, this new growth was extremely delicate and quite invisible except in certain plate areas and in favorable light. It appeared more like "angehauchten Glas" and developed much more slowly than the normal spreading HX19 culture. Other important aspects in the study of Braun and Schaeffer will be presented later in the appropriate sections, but we shall find it convenient to bear in mind this almost "invisible colony growth" emanating from the O type culture.*

The extent of dissociative reactions in a culture is important, whether in single colonies or in broad surface growths. The beginnings may show merely as areas of surface depression, sometimes central, sometimes marginal (Hadley [225], [226]), in which otherwise opaque (S) growth becomes translucent or even transparent over a sharply demarked area. There may be one or many such dissociation centers. Sometimes they are shallow and temporary; but more frequently they are progressive with increasing age of the culture; and in a few instances as in B. anthracis (Preisz,[393] Katzu [276]), in B. pyocyaneus (Canzik,[89] Hadley [225]) and in Monilia (Sonnenschien [448]), develop into well marked erosions which may within a week's time consume nearly the entire colony or culture mass. Particularly in the case of B. anthracis and Monilia the normal culture is replaced in the eroded areas by a dull, wrinkled and highly tenacious growth. The case of B. pyocyaneus I have described in detail elsewhere.

Suicide Cultures: There remains to be mentioned a striking mode of dissociation no doubt observed by many bacteriologists who have had laboratory experience, and this relates to the socalled suicide cultures, which have received mention by Collins [106] but have not obtained a significant place in the literature. Such cultures, after attaining within the first 18 to 20 hours a luxuriant growth, then seem literally to melt away within the space of another 12 to 18 hours until nothing is left but a thin, transparent film covering their former site. Such cultures are manifestly lytic and there is some evidence that they may be lysogenic. Because of the usual lack of corresponding S type cultures one is handi-

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* Referring to the terms H (double antigen type) and O (single antigen type) of Weil and Felix, we shall see subsequently that, despite the antithetical views of Arkwright and Goyle,[18] we are justified in regarding them identical with what will be termed the S (H) and the O (O) type cultures; R was presumably not observed by Weil and Felix, but may have been by Fejgin.[169]

capped in the study of the action of the filtrates. These cultures often fail to grow in beef broth. On agar, however, they may leave secondary colonies on the old culture site. Cultures from these colonies undergo no further lytic changes; they are resistant to the suicidal action. Regarding the position of these suicide cultures, they seem to occupy a place intermediate between those cultures manifesting the more common form of active dissociation and those which manifest frank, transmissible autolysis. Like both, their reaction is characterized by the elimination of S organisms and a generation of the R. Such cultures are of great interest and demand further study with reference partticularly to their lysogenic abilities. Certain aspects of this matter will be considered later.

To the above a few incidental points may be added. When dissociation occurs in a culture the growth energy always is markedly enhanced; we obtain, as I have pointed [226] out for B. pyocyaneus and some other cultures, a veritable proliferative growth accompanying or preceding the reaction. In connection with lysis accomplished by the bacteriophage in liquid mediums it may also be recalled that Bordet [61] has drawn attention to the necessity of "growth before lysis." D'Herelle [247] also has pointed out this reaction as a characteristic of all cells when attacked by a filtrable virus. Another minor point is the circumstance that, when the erosive type of dissociation occurs in agar slant cultures, it appears largely on the bottom half or two-thirds of the slanted surface. This also is true of the lytic areas caused by the bacteriophage, as shown by d'Herelle. We shall have reason later to return to these simple facts.

Dissociation in Liquid Medium.—It is also true that dissociation takes place in liquid mediums, and here even more actively than on solid surfaces (de Kruif,[120] Webster,[481] Mellon,[326, 327] Soule [450]). Sometimes the dissociation, accompanied by a greater or less loss of the S type organisms, registers itself by partial clearing. In other cases it can be detected only by enumeration of colonies when the broth cultures are plated or streaked on a solid medium. Here, however, the results may be misleading owing to the repressive action of the R type colonies on the S (de Kruif,[120] Pesch; [387] see also section 11). On such plates one may observe the presence or absence of R type colonies, or the relative proportion to those of the S type. Dissociation in liquid medium may proceed rapidly or slowly depending on its composition, the nature of the organism and its stage of development. According to Feiler [167] it proceeds more rapidly at 37 C. than at 22 C. Certain substances added to broth may hasten the process, while others may delay it (Webster [481]).

Rapid passage through an unfavorable medium increases the R type, while passage through a favorable medium increases the S type (de Kruif [120]). These aspects are considered more fully in section 11 on incitants to dissociation. Cultures which contain mixtures of S and R types have no doubt often been discarded because of suspected contaminations (Eisenberg,[150] Bernhardt,[49] Preisz [394]). In reality they may be mixed, but they are not on that account contaminated. By the time the dissociation has progressed sufficiently far to give a predominance of the R form, it usually manifests an agglutinative form of growth in broth, and will not remain suspended evenly in salt solution. Under these conditions it may still be kept in suspension by decreasing the salt concentration (Arkwright,[16] Shibley,[440] and others) to 0.42 or even to 0.21%. Although the R type agglutinates spontaneously, the precipitate is not always granular but may be flocculent. The granular precipitates are more commonly produced, as we shall see later, by another form of culture (P. B. White,[487] Goyle [206]). These variations in the form of growth, it may be noted, are not unlike what is often observed in the top and bottom growth of certain yeasts, among which I have little doubt it will be shown that a somewhat similar dissociation is also present.

So much insistence on the chief types of dissociates, S and R, is likely to leave the impression that these forms comprise all the significant participants in the reaction. Such a view, however, would be a great misapprehension of the actual situation. While it is indeed true that the "smooth" and "rough" forms are the most common and striking types, and seem to represent respectively the beginning and end of the reaction, a careful reading of Baerthlein and a consultation of many other works (Firtsch,[178] Eisenberg,[150] Bernhardt,[49] Arkwright,[17] Goyle [206]) impresses us with the fact that there are other forms of consequence. Baerthlein [30] often describes from three to five different colony types for the many organisms considered in his classical study of colony variation. Among these types it is always the S and R forms which stand out most clearly; but what of the others? Are they chance variations or are they of deeper significance? At present we cannot state, but the latter seems more probable. One type in particular appears in the literature with peculiar constancy, though usually mentioned as existing in small numbers. This type, which has been mentioned for many bacterial species, shows colonies that are large, round, regular, convex, fleshy and mucoid; they often resemble colonies of Bact. aerogenes. Microscopic examination reveals long filaments,

coccus forms and often giant cocci. It is fairly stable in propagation and some evidence suggests that it is the mother form of the R type; at least it is different from the true S and R forms. I mention this and the other variations only for the purpose of indicating before we proceed further that the problem of dissociation is by no means limited to the S → R "mutation," although this is at present its clearest aspect and one that will occupy our attention sufficiently. Sometime the relation of these other colony types (which it is scarcely feasible to discuss further at present) to the complete dissociative reaction will undoubtedly become clearer. At the present time all that we see clearly is that some of them are intermediate forms lying between S and R, and that a few of them present an extremely curious behavior when we attempt to cultivate them. To a more detailed consideration of this point we shall return in sections 5 and 12 of this paper.

This brief review of the general nature of the dissociative reaction is sufficient to indicate its main lines of action, and the sort of phenomena concerned. It will be the object of the forthcoming pages to expand into greater detail some of these issues which thus far have been only briefly mentioned. And, as we progress, we shall see that these apparently simple changes enter into many fields of bacteriological theory and practice.

4. RÉSUMÉ INDICATING THE EXTENT OF THE DISSOCIATIVE PHENOMENA, TOGETHER WITH THEIR PARALLEL TRENDS AMONG BACTERIA AT LARGE; SUGGESTIONS FOR TERMS OF REFERENCE TO PRIMARY DISSOCIATES

Extent of Phenomenon.—In the previous section I have dealt with some of the manifestations of dissociation but only in such a way as to indicate roughly its superficial mechanism and effects in ordinary cultures, without any attempt to look behind these surface phenomena to the deeper significance of the reactions. Since it would be impossible in the space available to give a summary of even a small part of the numerous references bearing upon the problem of dissociation, it is my intention to present in the pages immediately following, only a skeleton outline showing the implication of this phenomenon in a variety of bacterial reactions, but without in any case attempting to exhaust the subject. Subsequently I shall give a more detailed review of its bearing on certain aspects of bacterial behavior that are of greater practical significance in laboratory bacteriology. The following citations, arranged roughly according to subject, thus make no pretense of completeness, but are taken as representative of instances in which it seems

fairly certain that microbic dissociation was at work, although often not recognized as such at the time when the observations were made. As will be observed, the majority of the citations deal with microbic variations occurring either spontaneously or under the stimulus of modified environment. References to the source of the observations will be found in the literature list at the end of this paper.

(1) Microbic dissociation is concerned with the production of daughter colonies of a character manifestly different from the mother type on a background of old or dying culture; also when daughter colonies appear within mother colonies. The following examples may be presented:

B. anthracis (Preisz,[393, 394] Eisenberg,[149, 151] Pesch,[387] Katzu[276]).
B. coli mutabile (Neisser,[362] Massini,[320] Beneke,[45] Kowalenko,[286] Hubener,[269] Sobernheim and Seligmann,[447] Eisenberg,[154] Baerthlein[29]).
B. cyanogenes-lactis (Eisenberg[148]).
B. diphtheriae (Preisz,[393] Bernhardt,[49] Trautmann and Dale,[467] Enderlein[159]).
B. enteritidis (Eisenberg[148]).
B. fluorescens (Eisenberg[148, 153]).
B. herbicola (Beijerinck[42]).
B. indicus (Eisenberg[148]).
B. kielense (Eisenberg[148]).
B. malleus (Eisenberg[148]).
B. megatherium (Eisenberg[148]).
B. mycoides (Eisenberg[148]).
B. nitroxus (Beijerinck and Minckmann[43]).
B. prodigiosus (Eisenberg,[148] Baerthlein,[28] Enderlein[160]).
B. pyocyaneus (Eisenberg[148]).
B. ramosus (Eisenberg[148]).
B. septicemiae hemorrhagicae (Eisenberg[148]).
B. sporiferus (Eisenberg[148]).
B. subtilis (Eisenberg,[148] Soule[450]).
B. typhi-murium (Eisenberg[148]).
B. typhosus, paratyphosus, dysenteriae (Eisenberg,[154] R. Müller,[356] Baerthlein,[30] Burri,[88] Ledingham,[300] Thaysen,[460] Jacobsen,[265] Penfold,[385] Schröter and Gutjahr,[437] Gildermeister,[198] Morishima[352]).
Bact. pneumoniae (Eisenberg,[153] personal observation).
Corynebacterium diffidens (Enderlein[160]).
Diplococcus gonorrheae (Atkin[22]).
Diplococcus meningitidis (Bernhardt,[49] Atkin[21]).
Micrococcus albus and aureus (Lehmann,[301] Engelland, according to Enderlein;[160] Eisenberg[148]).
Sarcina aurantiaca (Eisenberg[148]).
Sarcina lutea (Eisenberg[148]).
Sarcina pulmonum (Eisenberg[148]).
Spirillum albensis (Eisenberg[148]).
Spirillum Finkler-Prior (Firtsch,[178] Preisz.)
Streptococcus fecalis, mitis, salivarius, hemolyticus (Faith Hadley[409]).

Vibrio cholerae (Preisz,[393] Eisenberg,[150] Enderlein,[160] Balteanu[38]).
Vibrio metchnikovi (Eisenberg[148]).
Vibrio rumpell (Eisenberg[148]).

(2) Microbic dissociation is probably at work in the production of the often observed regular or irregular colonies in the following cases:

B. acidophilus (personal observation).
B. anthracis (Bongert,[55a] Preisz,[394] Wagner,[476] Baerthlein[28]).
B. avisepticus (Manniger[317]).
B. coli (Neisser,[362] Massini,[320] Baerthlein,[29] Eisenberg,[154] Bernhardt,[49] Prell,[396] Arkwright[16] and others).
B. cholerae suis (Baerthlein,[27] Orcutt,[376] White[487]).
B. enteritidis (Baerthlein,[27] Topley and Aryton[464]).
B. diphtheriae (Corbett and Phillips,[110] Zupnik,[498] Schick and Ersettig,[430] Slawyk and Manacatide,[443] Bernhardt[49] and others).
B. dysenteriae (Steinhardt,[453] Baerthlein,[30] Arkwright[16]).
B. herbicola (Beijerinck[42]).
B. lactis-erythrogenes (Dyar[142]).
B. paratyphosus (Baerthlein,[27,30] Savage,[425] Breinl and Fischer,[71] Jordan[273]).
B. proteus (with special reference to spreading or restricted growth, Baerthlein,[30] Braun and Schaeffer[66] and others).
B. pyocyaneus (Baerthlein[28] and others).
B. tetanus (Hilda Heller[241]).
B. whitmori (Stanton and Fletcher[451]).
Bact. pneumoniae (Eisenberg,[153] Baerthlein[28]).

Also in the production of rough or smooth, soft or wrinkled colonies in many of the instances mentioned above; and in the following:

B. anthracis (Preisz[393] Wagner,[476] Gratia,[212] Nungester[409]).
B. avisepticus (Bernhardt[49]).
B. lepisepticum (de Kruif[119,120]).
B. proteus (Felix[172]).
B. of Schweinerotlauf (Wychelessky[493]).
B. subtilis (Soule[450]).
B. (thermophilic) (Koser[285]).
Streptococcus fecalis (Faith Hadley[499]).
Streptococcus hemolyticus, viridans (Cowan,[111] possibly Macchiati[314]).
Diplococcus pneumoniae (Baerthlein,[28] Blake and Trask,[52] Griffith,[215] Reimann,[405] Amoss[8]).
Vibrio cholerae (Celli and Santori,[93] Kolle,[282] Kruse,[292] Eisenberg,[150] Balteanu[38]).
Vibro proteus (Firtsch[178]).

Also in the peculiar colony form or culture growths of:

Actinomyces annulatus (Sector formation, Beijerinck[42]).
B. alkaligenes (Mellon and Yost[332]).
B. botulinus (Reddish,[402] probably).
B. butyricus (Schattenfroh and Grassberger,[427] Bredeman[68]).
B. diphtheriae (Kurth[294]).
B. gasterophilus (Sandberg[424]).

B. helicoides—"creeping colonies,"—(Muto[359]).
B. influenzae (Grassberger[207]).
B. lactis (Sandberg[424]).
B. pertussis (Bordet and Sleeswyk[64]).
B. pestis (Gotschlich,[204, 205] Dudschenko,[137] Shibayma,[439] Klein[279]).
B. (proteus) fluorescens (Jager[267]).
B. of Rauschbrand (Schattenfroh and Grassberger,[427] Meiszner[320 a]).
B. tuberculosis (Karwacki[507]).
Diplococcus gonorrheae (Atkin,[22] perhaps Lavrinowicz[299]).
Diplococcus meningitidis (Atkin[21]).
Micrococcus tetragenus (Eisenberg,[153] Wreschner[492]).
Sarcina lutea and other chromogens from air and water (Eisenberg[148]).
Staphylococcus albus, aureus, citreus (Lehmann,[301] Baerthlein,[28] Eisenberg[148]).
Streptococcus hemolyticus and viridans (Cowan,[111] Enderlein[160]).
Streptococcus fecalis (Faith Hadley[499]).
Vibrio Finkler-Prior (Firtsch[178]).
Oïdium albicans (Draper[502]).

(3) Microbic dissociation is present in the curious erosive phenomena observed in slant agar cultures or in plate colonies of the following organisms:

B. anthracis (Preisz,[393] Pesch,[387], Katzu[276]).
B. cereus (Andervont and Simon[9]).
B. paratyphosus (Gildermeister[198]).
B. pyocyaneus (Canzik,[89] Blanc,[53] Quiroga,[399] Sonnenschien,[448] Hadley[224] and others).
Micrococcus aureus, probably (Seiffert, on authority of Sonnenschien[448]).
Monilia (Sonnenschien[448]).
Unknown bacteria from air and water (Hadley[226]).
Also probably in all "suicide" cultures.

(4) The results of the dissociative reaction are shown in the non-agglutinative, or the spontaneously agglutinating growth of many bacteria in broth or serum, as in the following cases:

B. anthracis (Markoff,[319] Wagner,[476] Gratia[212]).
B. cholerae-suis (Orcutt,[376] White[487]).
B. diphtheriae (Corbett and Phillips,[110] Zupnic,[498] Slawyk and Manicatide,[443] Bernhardt[40]).
B. (diphtheroids) (Mellon[327, 328]).
B. dysenteriae (Steinhardt,[453] Benians,[46] Arkwright,[16] Arkwright and Goyle,[18] Zoeller[497]).
B. enteritidis (Topley and Aryton[464]).
B. lepisepticum (de Kruif[119, 120]).
B. melitensis, paramelitensis (Bassett-Smith,[39] Et. Burnet[85]).
B. paratyphosus (Weil and Felix,[484] Baerthlein,[27] Arkwright[16]).
B. subtilis (Soule[450]).
B. typhosus (Nicolle,[366, 367] Savage,[425] Steinhardt,[453] Moon,[351] Teague and MacWilliams,[458] Ishii,[262] Arkwright,[16] Arkwright and Goyle,[18] Feiler,[167] Burnet,[87] Gardiner and Walker,[196] Krumwiede,[288] Goyle[206]).

Bact. pneumoniae (Baerthlein,[29] Hadley[225]).
Diplococcus pneumoniae (Reimann,[405] Amoss,[3] Takami[457]).
Streptococcus viridans and hemolyticus (Mary Cowan[111]).
Streptococcus fecalis (Faith Hadley[499]).

Also in the phenomenon of the double and single antigen:

B. aertrycke (Furth[190]).
B. cholerae-suis (Smith and Reagh,[444] Furth,[190] White[457]).
B. dysenteriae (Arkwright,[16] Breinl,[69] Arkwright and Goyle[18]).
B. enteritidis (Gruschka,[217] Arkwright and Goyle,[18] Goyle[206]).
B. paratyphosus (Weil and Felix,[484] Furth,[188] Breinl and Fischer[71]).
B. pertussis (Bordet and Sleeswyk,[64] Krumwiede, Mishulow and Olden-busch[290]).
B. proteus X19 (Weil and Felix,[483] Braun and Salomon,[67] Braun and Schaeffer,[66] and many others).
B. typhosus (Joos,[272] Furth,[191] Arkwright and Goyle,[18] Goyle[206]).
Vibrio comma (Balteanu[35]).

(5) Microbic dissociation is observable in the course of "adaptation phenomena" as shown by organisms grown in broth containing antiseptics or other unusual substances:

Gentian violet.

B. coli, B. typhosus (Ainley-Walker and Murray[478]).
B. coli, Bact. aerogenes, B. lactis (Esther Stearn[452]).

Malachite green or brilliant green.

B. coli (Loeffler,[307] Revis[403]).

Sodium acetate, or oxylate.

B. coli, B. typhosus (Penfold,[385] Burnet[87]).

Bile.

Diplococcus pneumoniae (Reimann,[405] Amoss[3]).
B. coli (Adami, Abbott and Nicholson[1]).

Mercuric chloride and cadmium nitrate.

B. prodigiosus and other organisms (Wolf[490]).

Potassium bichromate.

B. prodigiosus and other organisms (Wolf[490]).
B. anthracis (Chamberland and Roux,[94] Surmont and Arnould[455]).

Phenol.

B. anthracis (Chamberland and Roux[94]).
B. coli (Malvoz,[316] Villinger,[475] Lommel[511]).
B. dysenteriae (Arkwright and Goyle,[18] Goyle[206]).
B. enteritidis (Goyle[206]).
B. proteus (Braun and Schaeffer,[66] Braun and Salomon[67]).
B. typhosus (Feiler,[167] Goyle[206]).
Vib. cholerae (Balteanu[35]).

Urea

> B. coli, B, typhosus, B. pyocyaneus, B. enteritidis (Wilson[485]).

Sodium chloride—in high concentrations.

> (Matzuschita[321]).

Saliva.

> B. coli (Adami, Abbott and Nicholson[1]).

(6) Microbic dissociation is concerned in the loss or the gain of capsules by the following organisms and presumably in others:

> B. anthracis (Chauveau and Phisalix,[97] Preisz,[393] Hess,[250] Bail and Flaumen-haft[34]).
> B. avisepticus (Manniger[317]).
> Bact. ozenae (Eisenberg,[153] Hadley[225]).
> Bact. pneumoniae (Wilde,[485] Beham,[40] Eisenberg,[153] Toenniessen,[462] Baerth-lein,[30] Hadley,[225] Julianelle[505])
> Bact. rhinoscleromatis (Eisenberg,[153] Hadley[225])
> Diplococcus pneumoniae (Kruse and Pansini,[293] Neufeld,[363] Eyre, Leatham and Washburn,[163] Laura Stryker,[454] Yoshioka,[494] Blake and Trask,[52] Griffith,[215] Reimann,[405] Amoss[8]).
> Sarcina tetragena (Eisenberg,[153] Wreschner[492]).

(7) Microbic dissociation is operative in many, and perhaps all, of the slow or rapid changes in the virulence of pathogenic cultures in vitro, and it may be equally in the bodies of immune or naturally refractory animals. The following cases may serve as examples:

> B. anthracis (Pasteur,[383] Chauveau and Phisalix,[97] Chamberland and Roux,[94] Hess,[250] Preisz,[393–4–5] Ascoli[20] and many others).
> B. avisepticus (Manniger[317]).
> B. cholerae-suis (Orcutt,[376] White[487]).
> B. diphtheriae (Roux and Yersin,[416] Corbett and Phillips,[110] Hewlett and Knight,[251] di Martini,[125] Zupnic,[498] Lessieur,[302] Haven,[238] Baerthlein,[28, 30] Bernhardt,[49] Schmitz,[434] Heinemann,[239] Goodman,[200] Crowell[114]).
> B. dysenteriae (Steinhardt,[453] Arkwright,[16] Fejgin[168]).
> B. enteritidis (Topley and Aryton,[464] Goyle[206]).
> B. lepisepticum (Bernhardt,[49] de Kruif[119, 120, 121]).
> B. paratyphosus B (Baerthlein,[27, 80] Jordan[273-4]).
> B. pertussis (Bordet and Sleeswyk[64]).
> B. pestis (Gotschlich,[204] Dudtschenko[137]).
> Bact. pneumoniae (Eisenberg,[153] Baerthlein,[28] Julianelle[505, 506])
> Diplococcus pneumoniae (Charrin and Roger,[96] Kruse and Pansini,[203] Neufeld,[363] Friel,[185] Stryker,[454] Yoshioka,[494] Blake and Trask,[52] Griffith,[215] Reimann,[405] Amoss[8]).
> Micrococcus tetragenus (Eisenberg,[153] Wreschner[492]).
> Streptococcus viridans, and hemolyticus (Charrin and Roger,[96] Cowan[112, 113]).
> Vibrio cholerae (Hamburger,[231] Ransom and Kitashima,[401a] Shousha[515]).

Also in changes in toxicity or toxigenic power of cultures:

B. diphtheriae (Roux and Yersin,[416] Goodman,[200] Bernhardt,[49] Crowell[114]).
B. dysenteriae (Fejgin,[168] Goyle[206]).
B. botulinus—perhaps (Reddish[402] and others).

(8) Microbic dissociation underlies the changes in type of organisms and in their biochemical and serological properties when cultures are grown in, or otherwise submitted to the action of, immune serum (homologous); or sometimes to normal serum if germicidal for the species concerned. This has been indicated in the following cases:

B. anthracis (Metchnikoff,[345] Sacharoff,[418] Sawtschenko,[426] Behring and Nissen[41]).
B. cholerae-suis (Metchnikoff[347]).
B. dysenteriae (Hamburger,[231] Steinhardt[453]).
B. diphtheriae (Bernhardt[49]).
B. subtilis (Soule[450]).
B. tuberculosis (Karwacki[508]).
B. typhosus (Nicolle,[366, 367] Steinhardt,[453] Eisenberg,[147] Rosenthal,[413] Park and Williams,[382] Saquépée,[421] Paul Th. Müller,[355] R. Müller,[356] Feiler,[167] Morishima[352] and others).
Bact. pneumoniae (Julianelle[505]).
Diplococcus pneumoniae (Charrin and Roger,[96] Stryker,[454] Clough,[102] Yoshioka,[494] Griffith,[215] Reimann,[405] Amoss[8]).
Streptococcus viridans and hemolyticus (Yoshioka,[494] Cowan[111]).
Vibrio comma (Ransom and Kitashima,[401a] Hamburger[231]).

Or when organisms are grown in tubercular, pleuritic fluid:

B. tuberculosis (Karwacki[508]).

Or when organisms are grown in urine, as in the case of:

B. coli (Horrocks — cited by Gurney-Dixon,[220] Wilson[485]).
B. typhosus (Hamburger and Czickeli[232]).

Or when they occur in naturally infected urine (as in urethritis):

B. coli (Archard and Renault,[11] Ali-Krogius,[2] Sörensen[449] and many later workers).*

(9) Dissociation is concerned with the spontaneous agglutination of many kinds of bacteria in 0.85% salt solution (and often in even less concentrated solutions) as shown by Nicolle[366] in 1898 for B. typhosus, and later by many others (Savage,[425] von Lingelsheim,[304], and others). In later years the subject has received closer study for members of the

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* It is impossible within the scope of this review to consider the voluminous literature relating to the variants of B. coli and B. typhosus occurring in urethritis and related infections. The majority, however, reveal modified forms, morphologically, culturally, biochemically and serologically. Among these the R types can always be detected in abundance, and with them often the intermediate O forms. These are to be found particularly in carriers or in convalescents and have been reported by Fletcher[179] and Gildermeister[198] (for B. paratyphosus), by Lacy[295] (for B. dysenteriae Shiga) and by Krumwiede and collaborators[288] for several paratyphoid strains. In typhoid carriers similar strains of B. typhosus have often been reported.

colon-typhoid-dysentery group (Arkwright [16]) ; for B. cholerae suis (White [487]) ; for Bact. lepisepticum (de Kruif [119, 120] Webster [481]) ; for streptococci (Cowan,[111] Shibley [440]), for certain diphtheroids—together with general considerations of the subject in relation to pleomorphism (Mellon [341]) ; also for Vibrio comma (Balteanu [38]).

(10) Microbic dissociation is concerned with the serological differences in cultures coming from different colonies in a single pure line as shown in:

B. cholerae-suis (Andrewes,[10] Orcutt,[376] White [487]).
B. dysenteriae (Baerthlein,[30] Arkwright,[16] Arkwright and Goyle,[18] Goyle,[206] Ørskov and Larsen,[513] Benians [46]).
B. enteritidis (Schütze,[436] Topley and Aryton [464]).
B. lepisepticum (de Kruif [119]).
B. paratyphosus B (Sobernheim and Seligmann,[446] Baerthlein,[30] van Log-hem [308]).
B. pertussis (Krumwiede, Mishulow and Oldenbusch [290]).
B. proteus (Weil and Felix,[483] Braun and Schaeffer [66] and others).
B. subtilis (Soule [450]).
Diplococcus meningitidis (Griffith [214]).
Diplococcus pneumoniae (Griffith,[215] Reimann,[405] Amoss [5]).
Streptococcus viridans and hemolyticus (Yoshioka [494]).
Vibrio comma (Balteanu [38]).

(11) Dissociation is often, and perhaps always, involved in the changes in biochemical or fermentative reaction occurring in pure line cultures, or in cultures from isolated colonies. It is manifested in changes in gelatin liquefaction in B. proteus (Braun and Schaeffer,[66] Baerthlein [30]), in Vibrio proteus (Vibrio Finkler-Prior) (Firtsch [178]), in Vibrio comma (Balteanu [38]), in B. subtilis (Soule [450]), in B. anthracis (Wagner [476]) ; also in the coagulation of milk in the case of B. proteus (Baerthlein [30]) ; also in changes in gas production in B. coli (Revis,[403] Sörensen,[449] Arkwright [16]) ; also in pyocyanin production in B. pyocyaneus (Hadley [224]) ; also in toxin production in B. diphtheriae (Roux and Yersin,[416] Corbett and Phillips,[110] Bernhardt,[49] Enderlein,[160] Crowell [114]) ; also in acid production in B. diphtheriae (Roux and Yersin,[416] Goodman,[200] Bernhardt [49] and others) ; in toxin production in B. dysenteriae (Fejgin [168]) and perhaps in B. botulinus (McIntosh and Fildes,[322] Reddish,[402] Hall [230] and others) ; also in fermentation reactions of B. coli mutabile (practically all the workers mentioned under the heading of "daughter colony formation"). Probably also in many other changes in fermentation reaction in which the presence of the dissociative reaction has not been observed in respect to correlated colonial or cultural variations.

(12) Dissociation is probably operative in the splitting of pure line yeast cultures as observed by Hansen, Beijerinck, Saito (from Tanner[456]), Baerthlein and others. Perhaps in the adaptation experiment to sodium fluoride of Fulmer and others. It may. occur in the "clumpings" of yeast cells in cultures as reported by Effront (from Tanner[456]), Fulmer[187] and others; also in the loss of sporogenic function and secondary colony formation in Sch. octosporus as reported by Beijerinck.[42] It also may play a part in the "top" and "bottom" yeasts of Hansen.[223] It is unquestionably present in the curious erosive reaction observed by Sonnenschien[448] in a culture of Monilia from the throat. It may be recalled that colonies of yeast often show the secondary colony formation observed among the bacteria (Beijerinck[42]).

(13) Dissociation and its results in the generation of the R type culture is responsible for the rapid or gradual acquisition of mild resistance toward the lytic influence of the bacteriophage, as is indicated in several of the studies of Bordet and of Gratia, as also by many experiments of my own. In this relation it occurs most commonly in cultures which have been carried for long periods in liquid mediums without frequent transfer. It underlies the phenomenon of "serological cosmopolitanism" (Schütze,[436] Goyle[206]) and similar heterologous serological affiliations as suggested by the work of Stryker,[454] Neufeld,[363] Yoshioka[494] and Reimann[405] for the R type pneumococcus and by the study of Torrey and Buckell[466] for their gonococcus strains; also by Julianelle[506] for Friedländer's bacillus; it also probably concerns the "convergence phenomenon" as exemplified by work of Esther Stearn[452] on intestinal bacteria under the influence of gentian violet. It unquestionably furnishes the basis for the "major" and "minor" agglutinins recognized in many groups of bacteria. Evidence supporting these last conclusions, and involving the S, O and R antigens, is presented in later sections of this paper.

(14) Microbic dissociation is probably connected with the generation of filtrable forms of bacteria derived from either normal cultures (or blood); pathologic tissues (or exudates); or cultures under the influence of the lytic principle which, as we shall see later, may act in the capacity of a dissociating agent. The following instances may be mentioned under their respective headings:

Filtrable forms of bacteria not known to be related to the presence of the lytic agent.

B. cereus (Andervont and Simon[9]).
B. (diphtheroid (Mellon[325]).

    B. fusiformis (Mellon[337]).
    B. of Johne's disease (Morin and Valtis[354]).
    B. of Schweineseuche (Lourens[313]).
    B. pestis (Et. Burnet[500a]).
    B. tuberculosis (Fontés[182] Valtis,[470] Durand,[138] Durand and Vaudremer,[139] Arloing and Dufourt,[19] Veber[473]).
    B. typhosus (Almquist,[3] Friedberger and Meissner[184]).
    Diplococcus meningitidis (Hort[255]).
    Leptospira icteroides (Noguchi[375]).
    Spirochaeta recurrentis (Novy and Knapp,[374] Breinl and Kinghorn,[72] Nicolle and Blanc,[369] Wolbach[489]).
    Spirochaeta elusa and biflexa (Wolbach[489]).
    Streptococcus—from encephalitis (Rosenow,[412] Evans[161, 162]).
    Streptothrix—(Mellon[325a]).

Filtrable forms of bacteria related to the presence of the lytic agent.

    B. coli (Izar,[264] d'Herelle,[246] Hauduroy,[235] d'Herelle and Hauduroy,[249] Tomaselli[463]).
    B. dysenteriae Shiga, Flexner and Hiss (d'Herelle,[246] Hauduroy,[234, 235] d'Herelle and Hauduroy[249]).
    B. gallinarum (d'Herelle[248]).
    B. pestis (d'Herelle[248]).
    B. typhosus (Hauduroy,[234, 235] d'Herelle and Hauduroy,[249] Fejgin[171]a]).
    Staphylococcus albus and aureus (d'Herelle and Hauduroy[249]).
    Organisms from river water (Hauduroy[236]).
    "Gonidial forms" of various bacteria (Miehe,[349] quoted from Enderlein[160]).

From this brief review of the extent and multiplicity of bacterial reactions with which it seems probable the phenomenon of dissociation is in some way related, we may conclude it is common not only among diverse types and genera of the lower bacteria, but among some of the higher forms as well. Wherever it occurs it is usually accompanied by changes in cultural growth, colony form, morphology of the cells, biochemical reactions, serologic reactions, antigenic power, virulence and resistance to injurious conditions in general; also to the influence of the bacteriophage.

The Existence of Parallel Trends in the Dissociation Process.— Having now outlined the sort of changes that characterize the phenomenon of active dissociation, it is of interest to observe the extent to which similar types of change may occur in bacteria at large and the degree to which they may run parallel through different, and sometimes distant, bacterial groups (Bernhardt,[49] 1915). It has already been intimated by the literature cited that such cases are numerous; indeed that some form of dissociation seems to be characteristic of nearly all bac-

teria and perhaps of all single celled organisms.* But it is also important to observe that parallel trends of dissociation are frequent, not only among closely related bacteria (B. coli, B. typhosus, B. dysenteriae), but also among bacteria belonging to diverse groups (B. coli, B. anthracis and Vibrio cholerae). In other words there exists a high degree of correlation in the frequency-distribution of characteristics belonging to the mother types, and a similar high degree of correlation in the distribution of characteristics appertaining to the daughter types, whatever the bacterial species may be. Without attempting to present a complete list of the many characters which run parallel for many organisms, and without meaning to imply that there are not exceptions to the character correlations presented, I may cite the following instances as supporting the point. The evidence for these cases, together with their proper references, will be found in various sections of this work.

CHARACTERISTICS MOST COMMONLY ASSOCIATED WITH
"NORMAL" AND "MUTANT" TYPES OF CULTURE

S, NORMAL TYPE	R, MUTANT TYPE
Homogeneous clouding in broth.	Agglutinative growth in broth.
Normal suspension in 0.8% salt sol.	Sedimentary suspension in 0.8% salt sol.
Fair, conservative growth on agar.	Often expansive growth on agar.[1]
Colonies smooth, regular, convex.	Colonies rough, irregular, flat.
May generate secondary colonies.	Seldom generates secondary colonies.
Agar growth soft, opaque.	Agar growth harder, translucent.
Agar growth fluorescent.	Agar growth seldom so fluorescent.
Agar growth pyocyanogenic	Agar growth nonpyocyanogenic
In motile species, active.	In motile species, nonmotile.
Possessing distinct capsules.	Noncapsulated.
Biochemically more active.	Biochemically less active.
Carries double antigen (S and O).	Often pure R; may have some O or S.
Generates the "specific soluble substances."	Lacks the "specific soluble substances."
Flocculogranular precipitate in serum.	Flocculent precipitate only in immune serum.
If a pathogen—virulent (or toxic[2]).	Slightly or nonvirulent (nontoxic[2]).
More common in active disease.	More common in carriers and convalesc.
More common in acute infections.	More common in chronic infections.
Sensitive to aging.	More resistant to aging.
Sensitive to bacteriophage.	Less sensitive to bacteriophage.
Represented by freeliving forms.	Product of adaptations.
Cells of "normal" morphology.	Tendency to short rods and cocci.
Transformed to O or R in S immune serum.	Not transformed in S immune serum.
Not transformed in R immune serum.	Transformed to S in R immune serum.[3]
Resistant to phagocytosis.	Susceptible to phagocytosis.

[1] Apparent exception in case of B. proteus.
[2] Relation to toxicity established only for B. diphtheriae and B. enteritidis; suggested for B. dysenteriae Shiga and for B. botulinus.
[3] Established for B. subtilis (Soule [450]).

In a general way in all bacterial groups, but especially within a single group, such as the colon-typhoid-dysentery (about which our knowledge of dissociation phenomena is most complete) the distinctive characteristics are not, at least in their fullest expression, interchangeable between the mother and the daughter types. As many writers have pointed out,

* Note Middleton [348] on Stolonychia, Jennings [265] on Difflugia, also Hansen, [233] Beijerinck [42] and Saito (see Tanner [456]) on yeasts, and Sonnenschien [448] on Monilia.

however, there often occur intergrading variations so as to produce cultures of "mixed" type; or, as P. B. White [487] has phrased it, variations in the "degree of roughness."

In connection with any attempt to discover these differences in the components of a given culture it also should be pointed out that some of the characteristics of the "mutant" form may not be discoverable at once in the new culture, but only after a period of growth, or sometimes after several selections of the most promising of the daughter colonies which have been continued on the new medium or in the new environment. The process is often gradual, although in other instances the dissociation may complete itself in the production of the R type with astounding suddenness. As a rule, the products of dissociation are found most frequently in old cultures, as shown by Firtsch [178] as early as 1888 for Vibrio proteus.

Although many exceptions to the character correlations mentioned in the foregoing tabulation may occur (and the number may increase with further study), they cannot alter the general truth of the circumstance that each group of closely related organisms is characterized by a set of mutation-like changes which accompany the phenomenon of dissociation in all the species of that group. Other bacterial groups may manifest "mutational" changes, some of which involve quite different features. If there exist any mutational trends that seem to be common property of nearly all the species and groups on which data are available, they are the changes relating to the following characteristics: assumption of a sedimentary or spontaneously agglutinative form of growth; change in size, form, color and consistency of colony; loss or modification in antigenic power and agglutinability; loss or diminution in virulence; increased resistance to unfavorable conditions of environment, including the action of starvation, antiseptics, heat, desiccation and the lytic principle. These are largely constant for all dissociating cultures so far as reported to the present time.

In the present attempt to emphasize the two chief types of culture and the usually parallel trends in the dissociative process as just described, the point must not be obscured, however, that the "mutant form" is not a unit, but comprises a variable group, the components of which may vary from one another while at the same time manifesting the mutant or group characters. Whether the different R types produced by different means from the same sensitive culture possess essential differences is a point on which we possess little knowledge, but one

which will be considered later (section 13) and with special reference to similar variations produced by the action of the lytic principle.

Suggestions for Terms of Reference to Primary Dissociates.—If all the variations or so-called mutations among bacteria were such that each species was a law unto itself, there would be little to be gained by making comparisons. But manifestly this is not the situation; for we have begun to see for the first time that, underlying these transformations occurring in very diverse bacterial types, there exist distinctly parallel dissociative trends. It is this mutation-like phenomenon which has already been termed "microbic dissociation," and for which I now wish to propose the term "spontaneous" or "active microbic dissociation," in order to differentiate it from what is apparently another sort of dissociation to be mentioned later.

In dealing with this problem, which as we shall see possesses some complexity in respect to the number and variety of cultures concerned, it is a matter of convenience, if not of necessity, to have available a terminology or mode of reference by which various bacterial stocks or strains can be referred to with some degree of exactness and precision. And the need of this will be seen to be greater when we come to consider the dissociative influence of the lytic principle in producing apparently similar changes of the primary types that have arisen through active dissociation. Such terms of reference as are to be recommended are, I believe, not likely to be of permanent value; but they may serve a useful purpose pending the time when we shall have acquired a deeper knowledge of the nature and meaning of the dissociative process. We may therefore consider a possible terminology sufficient for present needs.

A suggestion of what is demanded of a mode of reference can be obtained in part from a survey of the literature of the past three decades dealing intentionally or inadvertently with "mutations" and with some other apparent incongruities of bacterial behavior. Within this period bacteriologists, consciously or unconsciously, have given much information regarding the nature of the two fundamental culture types, the "mother" and the "daughter," as well as of a third culture type which we shall later come to recognize as a "transitional" form between the first two. It is the last two, but particularly the second, that have commonly been termed "mutants," and which have often received special designations by their discoverers. In the consideration immediately following, however, we shall confine our attention to the normal form and its "mutant" R. The intermediate type, which has been

observed less frequently (and for reasons which will subsequently be pointed out), will be considered later.

Although it is impossible to state accurately just when the two chief culture types made their appearance in the early bacteriological literature, it is safe to say that one of the first observations of the dissociative reaction was that of Firtsch[178] (in Gruber's laboratory) on Vibrio proteus (Sp. Finkler-Prior) in 1888. And, remarkably enough, this work still stands as one of the clearest expositions of the dissociative phenomenon so far as the cultural and morphological aspects are concerned. Neither in the study by Firtsch, nor in the later interesting study on B. lactis-erythrogenes by Dyar[142] in 1895, were any special terms of reference employed. Firtsch spoke of his different colony types as I, II and III, while Dyar referred to his normal culture as "smooth" and the "mutant" as "wrinkled." Bordet and Sleeswyk[64] in their study of variation in B. pertussis in 1910 employed the symbol MS (sang), and MG (gelose), in referring to the types of culture grown on a blood medium and on plain agar, respectively. Cecil Revis[404] in his experiments on the adaptation of B. coli to brilliant green and malachite green used the symbols A and B for the variants so produced; and Orcutt[376] used the same symbols for her variants of the hog cholera bacillus, adding AV and BV for later, secondary variants. Weil and Felix and their followers called their proteus X19 forms H and O. Von Lingelsheim[304] mentioned his typhoid variant as the Q form, used also by Gildemeister[198] for a form of B. paratyphosus. Joss[272] referred to his two chief antigenic typhoid types as α and β. Arkwright,[16] in studying members of the colon-typhoid-group, used S (smooth), R (rough), a usage which has been followed by Bordet and by Gratia for B. coli, and by Jordan[273, 274] for B. paratyphosus B. De Kruif,[119] however, referred to his two types of Bact. lepisepticum as D and G; and in this he has been followed by Webster[481] for the same organism as well as for B. typhi-murium. Both Griffith[215] and Reimann[405] in dealing with the dissociation of the pneumococcus employed S and R, while Amoss[8] for the same organism used C and Z. Cowan[111] in her study of streptococci followed the usage of Arkwright. Others have used certain descriptive terms: "regular," "irregular"; "homogeneous," and "agglutinating." More recently Ørskov and Larsen[513] have called their types of paradysentery culture V and B, with the further variants, M and Bu. Eisenberg,[150] and also Kolle[282] have referred to their two chief types of colony in Vibrio comma as "helle" and "dunkel," denoting thus the S and R (or O).

Besides these many other designations have undoubtedly been employed for new or unusual culture types.

In order to effect a common usage in dealing with these two common and manifestly significant bacterial types, I propose the general employment of S and R. If we let these stand for the opposed characters, "smooth" and "rough," or "sensitive" and "resistant," they have the advantage of appropriate reference, in the three most important languages in science, to the most frequent and most closely correlated pair of characters which exists for the respective types.* In addition to these instances X might be used, and could refer to the circumstance that its state and antigenic nature are unknown, as is most often the case when new cultures are encountered. Gratia [211a] has already suggested the use of O for such an original culture; but as we shall see, it seems more desirable to reserve this designation for another form, the intermediate, which will receive consideration presently. Of course an X type culture could be identical with S, or it might be either an intermediate, or an R type, of which (especially the latter) there are many in laboratory collections carried under the designation of normal culture.

One further term may be added to those already suggested. Sometimes a culture of the S type, although having given normal growth for a considerable time, suddenly and spontaneously presents the phenomenon of active dissociation, either on the edge of the fresh growth or at some point in the interior of the bacterial mass. The clearest instances of this phenomenon that I have observed are in B. coli, B. pneumoniae, B. lepisepticum (all marginal dissociations) and in B. pyocyaneus (central dissociation). Instances similar to the pyocyaneus dissociation, at least in certain macroscopic features, have been observed in B. anthracis and in Monilia, as already reported. When such a dissociation once begins, it may be perpetuated indefinitely in subculture so long as the medium remains favorable.

If, however, colony after colony and subculture after subculture are found to perpetuate this tendency to fall into autolysis at a certain point in their development, such a culture may be termed "lytic" or one of the L type. Bordet [60] has already used "lysogenic" for any culture which, though not manifesting lytic phenomena itself, is able to precipitate transmissible lysis in other cultures. In this sense the B. coli

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* As mentioned on a previous page, there are many instances which make it appear that the R type is not homogeneous, but may include cultures characterized by varying degree of roughness and correlated characters. If these observations are confirmed, the various R types might be referred to as R^1, R^2, R^3, etc., as will also be proposed for the resistant cultures arising from transmissible autolysis (section 13).

culture of Lisbonne and Carrére [305] was of the lysogenic or Lg type. From this point of view every S type culture is potentially lytic. At present, however, it seems that only the R type cultures are potentially lysogenic, but this point is by no means settled. Naturally a culture may be made up of two fractions and thus be both lytic and lysogenic. The need of further modifying these primary terms of reference will be pointed out in a later section dealing with somewhat similar dissociative changes effected by the bacteriophage or lytic principle.

Conclusion.—From the foregoing considerations it seems probable that the reactions which have been termed dissociative are to be observed at times in every bacterial species. Indeed, Dyar [142] in 1895 expressed the opinion that such colony variation as he observed in B. lactis-erythrogenes (and which we today recognize as being a fundamental manifestation of the dissociative reaction) could be seen in the majority of bacterial species; and, although he apparently had no conception of life stages in bacterial development, he saw nothing remarkable in the facts observed.

It now becomes clear that the dissociative reaction can be manifested by representatives of all the larger bacterial groups—aerobic and anaerobic, pathogenic and saprogenic, sporogenic and asporogenic— and among all morphologic types with the possible exception of the spirochetes, which as a rule have not been sufficiently studied in culture.

We have seen, moreover, that there exists a certain sort of parallelism in the trend of the dissociative phenomena in widely separated species. How close the parallelism is, and to what extent it is dictated by corresponding differences in the cells of the species concerned, remains to be ascertained. At least the parallel trends seem sufficiently well marked to make appropriate the differentiation of the chief culture forms into the S and R types, together with the less frequently observed intermediate, O, to be referred to on a later page. It is not to be assumed, however, that these terms are intended to serve as final designations in the sense that all S or all R organisms are alike morphologically or physiologically. They are merely intended to indicate that the culture concerned seems to become to a certain degree stabilized in a definite stage or type—sometimes highly transient, but at other times markedly permanent in its characteristics. In other words, these terms of reference are acknowledgedly superficial, referring only to certain gross appearances and reactions. With further study of the problem of dissociation they will doubtless give way to a more concise terminology.

5. FURTHER CONSIDERATION OF CHANGES PRODUCED BY THE DISSOCIATIVE REACTION: CULTURAL; MORPHOLOGICAL; MOTILITY; ENCAPSULATION; SPORULATION; CHROMOGENESIS

Between the mother (S) and daughter (R) types of culture there exist, as has been roughly intimated, important differences. While, in a general way, it has become a habit to differentiate these types on the basis of smooth or rough forms, a little experience with dissociating cultures indicates that, while these simple criteria are valuable, they may possess less significance in further differentiation than some others which have not as yet been sufficiently studied. I refer here to certain accessory morphological characteristics such as flagella, capsules and endospores; also to certain biochemical features including fermentative, antigenic, serological and immunological reactions. All of these will receive consideration in the course of this paper; but it is the chief object of the present section to describe the fundamental type differences as they relate particularly to the accessory morphological features, and to chromogenesis.

Cultural Characteristics of the Primary Dissociates.—When a culture that has undergone partial dissociation on solid or in liquid mediums, as outlined in the last section, is plated on agar, two (and sometimes three or more) different types of colony usually appear. Of these one is similar in most cases to the normal, mother culture (S), while the other is distinctly different (R). (The third type we shall consider in detail on a later page). The S colonies are usually round, regular, smooth, soft, glistening ("moist appearance") and sometimes highly fluorescent; while the R colonies are irregular, with broken or fimbriated margins, rough, granular or wrinkled, dull in luster, bluish or translucent, usually larger and occasionally tenacious to the medium. Of all these differences, two or more of which may appear in a given case, the most striking and constant relate to size, surface, and density of the colony. In some cases, however, and especially in first isolations, the differences in certain colonies are not clearly marked and there appear gradations between the clearcut rough and smooth forms. Moreover, as has been shown particularly for B. anthracis by Preisz,[393] Wagner,[476] Katzu [276] and others, and by Baerthlein [30] for many species, these colony differences may not be observable during the first 12 hours of growth. In other instances several days or weeks may be required for their differentiation (Baerthlein,[30] Penfold [335]). Toenniessen [462] has shown the same for variations in capsule formation in B. pneumoniae. White [487] particularly has recognized intergrading differences between the R and S types of Salmonella cultures and speaks

of the "roughest of the roughs." Such intergradations are not to be
wondered at when one recognizes that, although a colony may have
been founded by a single S type organism, dissociation in the colony
may begin at once, if the conditions are highly favorable; and, depending
on the progress atttained, may manifest varying pictures after successive
intervals. Further studies of such advanced colonies will invariably
show them to be mixtures of R and S, while other rough colonies may
yield apparently pure cultures of the rough type. Such cultures com-
pared with the S cultures are likely to be very constant, although they
may eventually show S inclusions. Thus, Arkwright [17] has been able to
isolate smooth type cultures of Shiga from a number of rough variants,
although these "derived smooths" differed in some respects from the
original S culture.

The plating from a culture that is undergoing dissociation often
gives mixed single colonies. These may be divided diametrically into
the two chief growth types, R and S; one-half the colony may be trans-
lucent, the other half opaque. Whether such pictures are determined
by the circumstance that a colony arises from two cells (one R and
one S), or whether dissociation started with the two-cell stage of the
colony cannot be ascertained. It is true, however, that cultivation from
each half yields its respective culture type. In some cases it should be
added, the division is not diametrical but the R type forms a gouge,
sector, or wedge-shaped area in the main colony mass, as if dissociation
had started in a cell at the margin rather than in the center of the
colony. This is often seen in dissociating streptococcus colonies in
which part of the margin is ragged while the balance is smooth (Faith
Hadley [499]).

When cultures of the type showing active erosive dissociations are
plated (B. pyocyaneus for example), at least three types of colony may
appear (Hadley [224]). These are: S type (nonlytic and pyocyanogenic);
R type (nonlytic and nonpyocyanogenic), and L type (lytic and highly
pyocyanogenic. Sometimes colonies such as the L type above have been
termed "lysogenic." But Bordet [60] has used this term with special
reference to cultures which, without themselves giving evidence of lysis,
are able to generate transmissible lysis in other homologous sensitive
cultures. It therefore seems preferable to designate as "lytic" a colony
which manifests lysis in itself, whatever may be its power of transmit-
ting the condition to fresh culture. It can of course happen that a
colony or culture can be both lytic and lysogenic, as Canzik [89] and
myself [224] have found to be the case in certain strains of B. pyocyaneus.

This was apparently not true, however, with the lytic anthrax colonies studied by Pesch,[387] Katzu [276] and others; nor apparently in the lytic culture of Monilia reported by Sonnenschien,[448] nor with the B. cereus dissociation of Andervont and Simon. The case made out for transmissible autolysis by means of lytic filtrates of B. pyocyaneus has been doubted by d'Herelle,[248] Sonneschien and others. Further reference to this matter is made on another page of this paper. Suffice it to say for the present that there is need of more detailed study of the action of filtrates of dissociating cultures, and particularly those of the acutely lytic type.

When transfers are made from smooth types of colony of intestinal organisms into broth, as Arkwright [16] and others have pointed out, the growth is uniformly turbid, as is also the case with suspensions in 0.85% salt solution. The rough colony transferred to broth, however, often forms a pellicle and yields a sedimenting or agglutinative growth at the bottom or sides of the tube. According to Arkwright, Cowan, Shibley [440] and others such cultures can be kept in suspension, for purposes of serological work, by reducing the salt content to 0.4 or even 0.2 %. Stable culture suspensions have been obtained by some workers by heating the antigen at 100 C. In broth the growth of the R type is usually slower than that of the S. When lytic (or lysogenic) colonies are inoculated into broth, sometimes no growth occurs. If it does occur it is likely to be of the agglutinative form and when examined later only organisms of the R type are present. Bordet [61] has called special attention to the rapid disappearance of cultures of the lysogenic type when inoculated into broth and left even for short times. He has also pointed out the varying proportions of S and lysogenic type colonies that may arise on plates under varying conditions of growth of S organisms in a lytic filtrate (bacteriophage).

In some instances, however, when colonies which manifest the cultural appearances of S are seeded into broth a condition of growth appears which might be termed "intermediate"; some agglutination occurs in a tube giving at the same time homogeneous clouding. This is due to a mixture of S and R, or of S and O, organisms, one or the other of which in repeated transfers usually gains the ascendancy. Although the colony appearance is usually a good criterion of the type of the organisms contained, it is not infallible. There is apparently greater chance that an old colony giving the superficial characters of the S type will contain some R than that a colony giving the appearance of R will contain S organisms.

In addition to the common R and S colonies, Arkwright [16] has men-
tioned another sort, "Rv," a variant from R. These colonies were small,
coherent and sticky. They arose from an R culture of B. dysenteriae
which, when grown in horse serum and then picked to broth, almost inva-
riably failed to grow. When transferred to agar the growth was thin and
endured for only two or three days. But after several short interval
subcultures on agar a culture was obtained that was still viable after seven
days. Further variants from R have also been reported by Orcutt [376]
for the hog cholera bacillus and for B. coli by Gratia.[211a] These data
show, as Arkwright has already pointed out and as was shown by Firtsch
for Vibrio proteus, that the R type itself may be capable of further
variation. To what extent there may occur variation among different
colonies of the S type is not known.

Regarding the nature of the R types of a single species, one
important question arises. Are they all the same regardless of the
manner of their production? The nature of the problem can be best
shown by reference to the work of Braun and Schaeffer [66] on the
O variants from Proteus X19 and X2. As stated earlier in this
review, these investigators produced the OX19 in two ways: one by
growth of HX19 on phenol agar, the other by growth of the same on
agar impoverished in the broth content. The O types so obtained
differed markedly from the H form from which they arose; but
morphologically and culturally they also differed from each other. We
shall see later that these modified types were in all probability not real
R forms of culture, although the one produced by starvation seems to
have been nearer the true R than was the strain modified by phenol.
Gratia [211 b] has shown the range of difference in R forms produced by
the action of the lytic principle in different concentrations. That dif-
ferent serologic reactions may exist in the case of different R forms
from the same culture has been shown by both White [487] and Arkwright.
These observations concern a problem of considerable interest about
which at present little may be said. It is sufficient for present purposes,
however, to indicate not only that the S forms differ from the R forms,
but also that the R forms differ among themselves. We shall find that
this is also true of the intermediates.

Dissociation in Relation to Cell Morphology and Motility.—In
our considerations thus far the influence of the dissociative process on
the individual cell has escaped notice. The effect in this respect, how-
ever, is no less significant than that on colony form or culture. We
must accept the truth of Baerthlein's [30] view that colony variations are

closely correlated with variations in cell morphology as well as with more essential physiological characteristics of the species. Omitting many references which are not found sufficiently related to known cases of dissociation to be of present value, we can still observe in the older literature some cases that merit citation.

Perhaps the most striking feature observed in the R individuals in different species is that some may be distinctly shorter and some longer than the cells of the S type. The S form of B. anthracis, for example, derived not from the "typical" anthrax colonies, but from the white, compact colonies, is composed almost exclusively of single rods, united at most into chains of two or three elements as pointed out by Preisz [393] in 1904 and confirmed by others. These organisms possessed something of the morphology of the cholera spirillum, that is, with rounded or slightly pointed ends and often a slightly curved body, quite different, as I myself have also observed, from the typical anthrax culture. The colonies of the R type, which were larger, more translucent, diffuse and of a dull luster (apparently conforming to the requirements of the "typical" medusa anthrax colony) gave quite a different picture. The cells were long, united into chains of considerable length or extended into filaments lying parallel for the most part and giving the common appearance of the "Medusa head" in the curled-edge colony. The ends of the cells were blunt or square cut, and never pointed. The cells and filaments were usually poor in spores. Sometimes, as Eisenberg [149] has shown, they may be quite absent. Whether the whiteness of the S colony as contrasted with the R is attributable to the richness of spores in the former, as Preisz believed, is uncertain, but on the whole doubtful, since the same point of differentiation occurs in the S and R colonies of nonsporeforming species.

In dealing with the relation of dissociation to cell morphology the work of Barber [37] in 1907 is of interest. Using the single cell isolation method that bears his name, Barber started about 140 pure line cultures of B. coli. The original organisms were selected for greater than average length. In all but one instance the population of each culture returned to the normal length of the strain as one would expect in the case of fluctuating variations. In one instance, however, he obtained a new race characterized by individuals of greater length. They also suffered some loss of motility and the cultures showed some changes from the normal type. This strain bred true for 32 months, undergoing frequent transplants to fresh medium. By similar methods of selection from other coli cultures two other "long races" were estab-

lished.   From the descriptions given it seems probable that Barber was
dealing with dissociative variants belonging to the O or early R type.
The extreme R type of B. coli is most commonly short, or even coccoid.

The dissociation of several cultures of B. subtilis has quite recently
been accomplished by Soule.[450]   For this organism he has obtained
and studied in considerable detail the two chief colony types.   He has
also isolated a third type, presumably an intermediate, which has as yet
not been studied sufficiently to permit of conclusions regarding its exact
nature or behavior.   Of the chief forms, one is smaller, whiter, more
compact, and possesses a distinctly glistening luster.   The other is
slightly larger, more translucent (at least in young colonies), diffuse
in manner of growth and possessing a dull surface.   The first type is
"domed" and about its edge the organisms are so arranged as to give the
typical appearance that Fraenkel has termed the "bayonet front" (quite
comparable with the "Krausköpf" anthrax colony of Preisz [393]).   The
second sort of subtilis colony, on the other hand, is thin and flat, with
a strong tendency for the outer fringe of colony growth to curl under,
exactly as in the "Medusa head" colony of B. anthracis.   It is the
culture yielding the first colony type that Soule has found dissociates
readily into the latter.   The reverse transformation, however, though
less common, was obtained in a number of ways (section 11, on
Incitants).   Soule concluded that the small, white colonies showing
motile bacteria represent the S type, while the larger, translucent
colonies, showing nonmotile bacteria, represent the R form.   The S form
occurred mostly in singles, doubles or short chains and were actively
motile, while those of the R type occurred in long chains and filaments.
Motility could be observed only in rare instances and then was sluggish.
When inoculated into broth the S type gave from the start a homo-
geneous clouding but with subsequent pellicle formation and partial
clearing.   The R form gave from the beginning an agglutinative growth.
Thus it appears that the S and R forms of B. subtilis may be clearly
differentiated by colony form, morphology and grouping of the indi-
vidual cells; also, as we shall see later, serologically.   Regarding the
change in motility it may be added that the complete transformation did
not occur in the first rough colony that was picked, but only after
several other colony selections had been made from the R stock.   The
first two or three selections yielded a much diminished rate of move-
ment which practically disappeared in the next few transfers.   In its
present state the culture is nonmotile, and highly stable.   The behavior

of the S and R forms of Soule's culture in the presence of normal and immune serums is presented in sections 9 and 11.

Evidence is presented later in this review demonstrating the identity of the H form of Weil and Felix's proteus X19 with the S type, and establishing the O form as an independent type not identical with the R. Although the O form of culture was first obtained by natural means, Braun and Schaeffer [66] produced it by growth on phenol agar, also by starvation (growth on agar with diminished nutrient substances). The morphology of the modified cells in this case thus seemed to depend on their manner of production. In the case of phenol agar the new cells were long, swollen and filamentous, while on starvation agar they took the form of minute coccobacilli, "not unlike Pfeiffer's bacillus." Neither type resembled the original HX19. Braun and Schaeffer add an interesting comment on what appears to them as an adaptive reaction in the respective changes: the reaction type to phenol (poisoning) is one which affords the greatest volume to the least surface, while the reaction type to impoverished agar (starvation) is one which affords the greatest surface to the least volume.

Although in the R cultures of B. coli and other members of the intestinal group there may often be recognized long or filamentous forms of the organism, especially in the earlier passages of R culture, the usual R type is a coccobacillus often approaching a coccus, as reported by numerous authors for B. coli, B. dysenteriae and B. typhosus (Arkwright [16]). This variation holds true whether the dissociation is produced in vitro by artificial means or occurs "naturally" in the urine in cases of cystitis, pyelitis, pyelonephritis, etc. (Mellon,[328] Zdansky,[495] Hamburger and Czickeli,[232] and others). It may be added that it was a considerable time before these atypical forms were recognized by serologic tests as related to B. coli; and as will be shown in section 9, even in such tests, the reactions are far different from those of normal B. coli. These variations in morphology remain constant over long periods. Similar types, it may be added, result from the action of the bacteriophage on a sensitive culture of members of this group (d'Herelle, Gratia). This subject will be considered in section 13.

One further point regarding the morphological aspects of the cell transformation from S to R is of special importance—namely, that it is not a sharp and direct change, but one that passes through an intermediate stage in which the variability in the morphology of the cells is much increased over that of the S type. This has been pointed out particularly by Feiler [167] in his report on the dissociation of B. typhosus,

but that the phenomenon occurs rather commonly, and perhaps in all cases, appears from much other evidence. Feiler, among other dissociation provoking methods, made use of phenol agar. The population of the original culture was fairly homogeneous in morphology, but on the first phenol agar slant he reports that many peculiar and bizarre forms, lacking motility, developed. These, however, disappeared after two or three passages on the same medium and thicker and plumper rods gradually came into prominence. This and other similar observations indicate, I believe, as will be mentioned later in greater detail, the existence of an intermediate culture type through which the culture passes in the course of transformation from the S to the R form, which we may doubtless regard as the end product of dissociation. It seems probable that many of the intermediate colony types that have been described are not necessarily mixtures of S and R but mixtures of R with the transitional form of culture, O, to be described later.

Turning now more fully to the relation of dissociation to motility we find as one of the clearest instances the change occurring in B. proteus in its passage from the H to the O form, as also reported by Braun and Schaeffer [66] in 1919. The passage of the H form of X19 of Weil and Felix on phenol agar, or on starvation agar, gave cultures which varied from the normal not only in cell morphology, as already mentioned, but also in motility and flagellar equipment. After one or two passages the loss of flagella was noticeable, but after many passages no flagella could be found on any organism. The loss was the same, whether the O form arose from phenol agar or from the "starvation agar."

Regarding the R type of various members of the colon-typhoid-dysentery group, Arkwright [16] has stated that they possessed little or no motility. Baerthlein [30] showed that the variant colonies of Vibrio cholerae contained only nonmotile organisms. Krumwiede and collaborators [289] showed the same for R, and mucoid, paratyphoid variants. Gratia,[211] on the other hand, stated that the S type of B. coli studied by him was nonmotile while the R form was motile. It is also to be noted that Gratia reported the R type to be more virulent for guinea-pigs; also less phagocytable. These findings are quite remarkable and require confirmation. I have found the R type of several B. coli strains all nonmotile.

Feiler [167] in 1920 gave a clear picture of the effect of dissociation on the motility of B. typhosus. The reaction was produced by the same methods employed by Braun and Schaeffer for the dissociation of

B. proteus. Cultures of the actively motile S type were grown on starvation agar, phenol agar and in homologous immune serum. Feiler observed that the starvation R strains differed the least from the normal type and the immune serum R strain differed the most. In all cases, however, the difference was increased by continuous passage on the respective media. The starvation R, though losing its motility, failed to lose all of its flagella, although the flagellar system was much damaged. The phenol R lost all motility and all trace of flagella. In both of these cases a return of the cultures to normal culture medium was followed by a return to the normal culture type and with it the normal flagellar apparatus. Continuous growth in immune serum, however, not only determined the loss of flagella but also produced an absolute R form which, when it was returned to normal medium, failed to revert to the normal flagellated S type. Feiler thus regarded it as a permanent modification. He developed particularly the conception of the double antigen hypothesis for his S type culture contrasted with the single antigen of the R. The explanation of the double antigen was developed by Feiler (as it was developed independently by Wege Weil, and by Felix and Mitzenmacher,[173] apparently without the recognition of still older observations on this point) on the basis of endoplasmic and ectoplasmic agglutinogens, the latter comprising mainly the flagellar protein.

These instances could be multiplied but a sufficient number has been presented to indicate that the dissociative reaction is ordinarily accompanied by deepseated changes in both morphology and motility. The R type is ordinarily nonmotile and nonflagellated. Whether there are other types besides O that are nonmotile but at the same time not R forms, cannot be stated. That the loss of flagella is a gradual process, however, is indicated by a number of reports, but most clearly by Braun and Schaeffer. Regarding the changes in cell morphology, apparently their appearance is not easily predicted, but depends upon the nature of the organism undergoing dissociation (for instance, B. coli compared with B. subtilis) and upon the nature of the stimulus to dissociation (for instance, phenol agar compared with starvation agar). In most nonsporebearing bacteria the direction of the change is certainly toward the foreshortening of the rod, with the consequent production of coccoid and even coccus forms. The types of B. coli found in cystitis, pyelitis and gallbladder infections serve as good illustrations of this point. The situation seems to be different, however, in the sporogenic forms such as B. subtilis and B. anthracis.

Dissociation and Encapsulation.—The same dissociative mecha-
nism operative with respect to cultural characteristics, cell morphology
and motility can be detected in the modification of other characteristics
of bacteria. We may take, for example, capsule formation exempli-
fied by Friedländer's pneumobacillus as it has been studied by Wilde,[485]
Eisenberg,[153] Baerthlein[30] and Toenniessen,[462] and more recently by
Julienelle[505] and myself.[225] Baerthlein as others just mentioned started by
plating out old cultures and detecting various colony types, among which
we can now clearly recognize the S and R forms, the former encap-
sulated, the latter not. In my own case I used mass cultures on agar
plates only a few days old and selected material from the translucent
wedge-shaped marginal invaginations which make their appearance
sooner or later along the free edges of the culture mass, and which are
made up almost exclusively of organisms of the R type. Selecting from
normal areas yields normal capsulated bacteria, characterized (when
plated) by a form of colony quite different from that of the R type.
One may however obtain the dissociation from plain broth cultures.
If we start with a culture fresh from the infected animal, or one grown
in a blood medium, we observe that the growth is highly viscous and
microscopical examination of stained films reveals heavy capsules on all
bacteria. If now we make passages through an impoverished broth
medium, such as extract broth or peptone water, we observe that the
viscosity diminishes with every transfer. If, however, at intervals in
this series, we examine microscopically a stained preparation, we do not
find that each organism has lost "a little of its capsule," but that some
organisms have lost none and others have lost all. If the transfers
through the impoverished medium are continued we reach a stage in
which all observable bacteria are noncapsulated. These represent the
secondary, resistant type; they have also lost their virulence. The same
result can be determined by streaking agar plates and selecting cultures
from the translucent marginal areas of completed dissociation in dis-
sociating colonies. Here again, therefore, we are apparently confronted
with no slow change overcoming equally all the organisms in the culture,
but with the sudden appearance of a "new" pneumobacillus type. It is
not necessary to state that these changes are reproducible in cultures
coming from single colonies, after several repeated platings; or that,
starting with a culture predominantly of the noncapsulated type, it is
possible, by return to a favorable medium, or by animal inoculation, to
regenerate the capsulated form in abundance. If the capsulated type has

been once lost, however, as in an apparently "pure" R type colony I find no evidence denoting the possibility of its return, although I have not studied the matter extensively.

Although the loss of capsulation accompanying dissociation in Bact. pneumoniae seemed to be accomplished quickly and decisively in the cases that I have studied, Toenniessen, on the contrary, has presented a different picture, demonstrating the loss of capsule by gradual steps. In order to enforce his changes he made use of chemical antiseptics and by this means was able to "fix" the degree of capsule formation in different grades—complete, intermediate, or slight—each grade remaining constant in further cultivation on a favorable medium. These results, while differing somewhat from my own findings, correspond well with the manner of loss of motility in B. proteus and in B. subtilis during dissociation. In addition they conform with the views of White, Arkwright and others regarding the existence of degrees in the expression of the "rough" characteristics in R cultures coming from different colonies.

In 1921, in an article dealing with the biological significance of capsules in Micrococcus tetragenus, Wreschner [492] made an important contribution to the phenomenon of dissociation in the sarcina group. The culture concerned came from the mouth and grew at first in the form of gray-white, slimy, convex colonies. In the course of further cultivation on ordinary agar the colonies lost their sliminess by degrees and came to resemble the colonies of a staphylococcus. Along with these colony modifications went changes in the morphology of the single cells. By the fourth transfer the capsules, previously abundant, had begun to disappear and by the twelfth transfer they had vanished. If platings were made of the culture in this stage the colonies were pure white, flat and, when young, somewhat smaller than the capsulated type. In the center was usually a depression. While the original colonies could be removed en masse by reason of their viscosity, the new form was butyrous. In broth the noncapsulated form showed much greater growth energy and, when planted in equal volumes with the capsulated, in 24 hours outgrew the latter "by eight to nine times." In plating such mixtures, sometimes hardly a capsulated colony could be discovered. Propagated in ordinary broth the new form was "absolutely constant," but reverted to the capsulated when blood was added to the medium. The capsulated type was highly virulent, while the noncapsulated form lacked virulence. (For further consideration of this point see section 8.)

Although Wreschner has given the clearest picture of dissociation in M. tetragenus, the case is also apparent from the earlier study of Eisenberg [153] in 1914. As in the majority of Eisenberg's studies on bacterial variation, he plated an old (40 day) agar slant culture. Two sorts of colonies appeared: One was large (2 to 6 mm.), round, even convex, slimy and half-transparent. The other was smaller at the start (0.5 to 2.5 mm.), even, round, porcelain white and opaque. The first colony type gave well capsulated packets and tetrads. The second type gave only noncapsulated forms, or organisms with "rudimentary" capsules. The capsulated form of culture was much more virulent for mice. After one month in broth or alkaline peptone water the capsulated type had become completely transformed to the noncapsulated, while under the same conditions of cultivation the noncapsulated organisms underwent no change.

In reviewing these observations of Eisenberg and Wreschner there can be no doubt that both were dealing with dissociation phenomena and that the capsulated and noncapsulated organisms represent respectively our S and R dissociates. Intermediate types (O) have not been observed. The subjects of reversion and virulence in capsulated bacteria are treated in greater detail in section 8 of this work.

In the case of the pneumococcus the capsulated nature of the S type and the noncapsulated nature of the R have been clearly pointed out by Griffith, Reimann, and by Amoss. I have noted the same phenomenon in both B. ozenae and B. rhinoscleromatis. The latter observation confirms the earlier report of Eisenberg for the same organism.

The relation of capsules to the chief dissociates of the anthrax bacillus as we at present understand them is not so clear as in the case of Friedländer and M. tetragenus. If it were permissible to reason from analogies, the situation existing among other capsulated bacteria would lead us to predict that the S type of anthrax would be capsulated and also virulent. We shall see that, although the virulent type of the organism is usually regarded as the capsulated form, according to our present interpretation the virulent form of anthrax is not the S but the R. But we may turn to review the actual evidence.

Although it might be possible to read into the older work of Chauveau and Phisalix [97] a delineation of dissociation, the picture is not clear so far as capsules are concerned. Suggestions of a correlation between capsules, virulence and nonphagocytability appear in the later studies by Löhlein,[309] Deutsch,[126] Donati,[132] Kodama [281] and Preisz.[394, 395] The observations of Preisz [394] (1911) are of special interest. For a number

of years previous to this date he had control of the Pasteur vaccines employed in anthrax immunization in Budapest. During this time he checked them for purity by the plating method. Occurring among the normal anthrax colonies on these plates he observed more or less constantly two abnormal colony forms which he at first took for contaminations, and the appearance of which made him hesitate to use the vaccines, either I or II. Further study of these new colony types, however, showed that they were in reality modified anthrax types. Of these the preponderant form was moist, glistening, transparent, "structureless" and slimy in varying degrees. When touched with the needle it would often spin into threads of 30 to 40 cm. Since he saw many normal colonies apparently becoming transformed to the slimy type, but since this form could also give rise to the normal, he termed it the "Uebergangsform." Although large animals could not be infected with this culture, small animals died, and from these he could recover normal culture. The degree of sliminess showed considerable variation; while some colonies were dense others were almost liquid in consistency. When in this form they did not give well isolated colonies on the plates but ran together into irregular groups and masses. These often appeared like "Schleimtropfen." The organisms in these slimy colonies were commonly capsulated but the degree of capsulation varied with the density of the sliminess of the culture. Cultures of greatest density showed bacteria surrounded with firmly adherent capsules, while those of more watery constitution showed organisms less definitely capsulated, as if the capsule substance had run off from the bacteria to produce an interbacterial slime. Of these cultures, as well as of the individual organisms, Preiz presents good photographs. They were found in both vaccine I and II, and perhaps represent the anthrax O types.

The second type of variant from the normal medusa form was represented by white colonies that were perfectly round and with even or slightly fringed edges. These colonies which one would be inclined to call the S type, not only by reason of their form but also from analogy with Soule's [450] results on the dissociation of B. subtilis, were not slimy and were composed of bacteria that were noncapsulated and entirely lacking in virulence. Even small laboratory animals failed to manifest infection, and when killed later no organisms of any kind could be found in their blood. This was true for the white colonies obtained from both vaccine I and II. It is apparent that this is the same type of colony described later by several investigators, particularly Bail,[32] Eisenberg,[149] Markoff,[319] Wagner [476] and Gratia,[212] but without clear reference to

capsule formation and with somewhat contradictory reports on the sub-
ject of virulence. Preisz was able later to duplicate these results by
heating normal virulent anthrax cultures and expressed the view that the
reason why earlier workers of the French school had not observed these
unusual culture forms was because they worked largely with broth cul-
tures, rather than with B. anthracis on solid culture mediums. It may
be added here, however, that the slimy cultures and the white cultures
of Preisz do not necessarily owe their existence to the heating of normal
virulent cultures. Mr. Nungester [499] in our laboratories has obtained, in
the course of colony selection in normal dissociation, cultures which are
in every respect identical with those described by Preisz. Of the fact
that they represent transitional forms between S and R there can be
no doubt.

In the work of Bail and Flaumenhaft [34] in 1917 we also find data
bearing upon the relation between capsules and type of organism in
B. anthracis. They found that, if a virulent culture was exposed to a
temperature of 42 C. for a period that just fell short of depriving it
of ability to form capsules, certain strains were produced which on
plating yielded a mixture of colonies. These we can easily recognize as
the S (Bail's a) and R (Bail's β), the latter being more virulent for
guinea-pigs. Neither form, however, showed a tendency to return to
the capsulated state. Hess [250] in 1921 described two forms of anthrax
colony and indicated the presence of capsules in both. The number of
capsulated bacteria was however less in the culture (Stamm III) which
we may regard as the S type. The rough colonies gave cultures in which
all of the organisms were more or less heavily capsulated. Continuous
cultivation in horse serum broth caused a loss of capsules in all cases.
Hess concluded that, for the development of capsules, a special stimulus
is required. For normal bacteria a weak stimulus may be effective, but
for modified bacteria "the stronger stimulus of the living body is
required." In other words, for modified bacteria, serum in vitro is not
effective. From these somewhat confusing results relating to capsules
in B. anthracis, we can perhaps make out that they stand in some rela-
tion to virulence, which itself, peculiarly enough, seems related to what
we have considered as the R type of culture. The whole problem of
dissociation in B. anthrax is in an unsatisfactory state and much in need
of further study.

From the foregoing considerations it is apparent that, although cap-
sule formation is often correlated with the S form of culture, it also
appears in the R type of anthrax. Our conclusions on this point, how-

ever, cannot be final. In addition, capsules often appear in the intermediate culture type lying between the extremes, S and R. As we shall see a little later, other bacterial species also manifest a transitional form showing the same slimy characteristics. The Q form of typhosus of v. Lingelsheim [304] may have been one of this sort since he emphasizes its slimy qualities; also the Q form of B. paratyphosus described by Gildemeister,[198] which was clearly in a lytic state. Other slimy cultures occurring in species which do not ordinarily produce definite capsules were seen by Ledingham [300] who suggested that the mucus-like substance might be related to the peculiar agglutination features also observed. Arkwright [16] reported sliminess for the assumed R type of certain Shiga cultures and was inclined to connect it with the presence of "swollen-looking, large, broad, irregularly-shaped bacteria, sometimes showing bud-like and branch-like processes. . . ." The sliminess, he attributed to changes in internal composition of the bacilli and not to capsule formation. These forms were probably mixtures of O and R, rather than pure R types. Such forms we shall see later often exist among the intermediates. It is safe to say that the "extreme R" is never capsulated. It may be added here that many cultures, arising as secondaries to the action of the bacteriophage, possess mucoid characteristics, but it has not been clearly shown that they are capsulated. Indeed, Kimura [278] has apparently shown that the B. coli from mucoid colonies produced by the bacteriophage, has no capsules but that the slime is a "secretion product." He believed this slime had the power of protecting the bacteria nonspecifically against the lytic principle. I [225] have shown, however, that, in Friedländer's bacillus, heavy capsules are no obstacle to bacteriophage action.

Dissociation and Spore Formation.—That nonsporogenic strains of bacteria can be isolated from sporogenic cultures has been recognized for many years, and the same is true for yeasts. Moreover, it is a matter of common knowledge that such strains, for a time at least, tend to breed true. In view of our present knowledge of dissociation, it might be suspected that this reaction is in some way connected with the phenomenon of sporogenic versus nonsporogenic types. This possibility indeed seems to have some support in the observations of Mellon and Anderson in 1919 [344] that spore substance and vegetative cell substance possess marked antigenic differences in the case of B. subtilis. The older literature on variations in sporeforming bacteria, particularly B. anthracis, although in no single instance revealing the entire truth of the situation, affords elements of evidence which are mutually com-

plementary and to a large measure confirmatory. These may there-
fore be reported in such form and order as to give a picture of the
situation as it exists at the present moment.

We have noted in many instances that the ability of a colony to
generate papillae or daughter colonies seems invariably to be correlated
with reactions of dissociative significance (Neisser,[362] Massini,[320]
Müller,[356] Thaysen,[460] Penfold,[386] Baerthlein,[30] Eisenberg [148] and
others). That such a phenomenon occurs in anthrax colonies was
shown certainly among the first, by Preisz [393] in 1904. He points out
typical and atypical strains, and presents the colony differences in detail.
He also states that in the case of weak spore formation the colonies
are blue (transmitted light), while in rich spore formation they are
whitish. In the larger, blue colonies, which seem to represent the more
typical form, there arose after a few days to a week small papillae
(Knötchen), "the size of a sand grain," and these increased in size to
form half-moon shaped colonies, yellowish-white in color and appearing
like contaminations. If numerous, they imparted an uneven appear-
ance to the colony. These "Knötchen" were found to be made up of
spore-forming bacilli, while spores were absent in the surrounding
culture; and Preisz concluded that they arose from the germination and
subsequent multiplication of the spores of bacilli of a new type and
different from the old culture (colony). They were shorter
and occurred largely as single cells or in short chains, whereas the
nonsporeformers were long, thin and united into chains of many ele-
ments. The ability of cultures to give the secondary (or even tertiary)
colonies was held for a long time in cultures characterized by slow and
rare spore formation; and such cultures were always blue or translucent
as compared with the whiter sporeforming cultures. Preisz found no
sporeless races but many in which spore formation was "almost lost."

In 1912 Eisenberg [149] reported studies on spore forming and spore-
less races of B. anthracis, dealing chiefly with the possibility of trans-
formation, one to the other type. He pointed out that in many anthrax
colonies, after 12 to 16 hours of growth on agar, a differentiation
occurred in which some became whitish with a glistening surface while
others were dull and eventually became bluish and transparent "as if
self-digested." Microscopically the whitish colonies showed chiefly
sporulating rods and free spores; and to this he attributed the white
appearance of the culture, as Preisz had done earlier. The second
culture type was composed mainly of organisms without spores or with
only a few. Such asporogenic cells, however, contained many "fat

globules" while the sporogenic cells lacked them; he therefore assumed that these granules were normally used up in spore formation. Signs of an autolytic process were often evident. With reference to the mutual transformation of the two types, it was found that neither rapid passages on agar, nor successive passages in guinea-pigs (apparently the organisms possessed some virulence), served to change a sporeless race into a sporogenic. Indeed the only successful method reported was to heat the asporogenic culture at 70 to 90 C. This procedure yielded a strain rich in spore production. He found it possible, however, to transform a spore race into a sporeless by five to 20 passages on glycerol agar, or by the addition of grape sugar to the medium; this last method, however, gave less constant results. Some sporeless races produced by the glycerol agar method are said to have remained constant. Eisenberg did not employ single cell isolation methods but he did take the precautions to make eight successive colony isolations preceding his study; and this circumstance lends special significance to his results.

The significant study of Wagner [476] in 1920 has been reviewed in another section and needs reference here only to the extent of recalling that he, as well as Markoff,[319] Baerthlein [28] and Gratia,[212] pointed out the two chief colony types of B. anthracis and also suggested the difference in sporeforming ability although this point did not receive special consideration.

Most recently Pesch [387] and also Katzu [276] have reported for B. anthracis a peculiar disintegration phenomenon simulating the action of the bacteriophage, but which they concluded was of different nature, although perhaps belonging to "the same group of phenomena." The appearance of the anthrax cultures, it may be noted, was similar to that pictured by myself [224] and by Sonnenschien [448] for B. pyocyaneus; by Sonnenschien for Monilia; also by Andervont and Simon [9] for B. cereus; and possibly by Lawrence and Ford [298] for B. megatherium. The phenomenon concerned macroscopic erosive action in colonies and in mass cultures. Pesch, working with B. anthracis, obtained the usual two types of colony from two-year old agar slant cultures. He stated, however, that there existed no difference in the morphology of the organisms nor in the extent of spore formation. Neither form was virulent for mice. He was able, however, to obtain the same dissociative reaction in several virulent cultures and regarded the changes as "related to variation phenomena."

The results of Katzu's study were more or less similar. He obtained two anthrax cultures, one a heated Pasteur "vaccine strain," the other

fully virulent.   The former culture, after standing for about two months
on sheep agar, showed round, bare areas ("Löcher") which penetrated
through the culture mass to the agar.   Over the floor of the pockets
was a thin, transparent film of growth.   Daughter colonies (secondaries)
were observed along the border of some of the larger colonies.   Whether
subcultures were made from the pockets themselves, or from areas of
culture between the pockets, the resulting cultures yielded similar
erosions.   I have demonstrated exactly the same circumstance for lytic
cultures of B. pyocyaneus.   Katzu showed, as Preisz had done much
earlier, that the pockets could be traced back to small granulations
("Kolonienknöpfen," Knötchen) in which a different type of organism
possessing marked capacity for autolysis was being generated, thus con-
firming the older and highly significant observation of Preisz on the
same point.   Katzu also described again the "Krausköpfähnliche"
(S type) colonies and pointed out that these did not contain erosions.
Although the fully virulent anthrax culture studied by Katzu did not at
first present these dissociative phenomena, growth of this culture at 42 C
induced similar erosive manifestations.   Attempts on the part of both
Pesch and Katzu to obtain a filtrable agent capable of producing similar
changes in normal culture were without success.   Katzu concluded that
the reaction studied merely involved the splitting off of a transparent
variant from the mother culture.

Other early contributions of interest bearing upon one aspect or
another of this problem are those of Altmann and Rauth [7] (1910),
Burri,[88] Phisalix [389] (1893), Sobernheim and Seligmann (in Kolle and
Wassermann) and Wolff.[490]   A good review of studies on the variability
of B. anthracis is given by Prigsheim.[397]

From this brief review of observations on dissociation in B. anthracis
with special reference to spore formation it seems probable that the
sporogenic and asporogenic cultures correspond respectively with the
two chief colony types: the first being small, whitish, round, smooth
and regular; the latter larger, bluish or translucent, somewhat rough
("matt") and irregular or spreading.   The latter form manifestly con-
stitutes the "typical" anthrax culture, giving the Medusa head colonies,
but the direction of the dissociative trend is clearly from the atypical to
the typical form, seldom the reverse.   These observations suggest that
the former (atypical) is the S type while the latter (typical) corre-
sponds with the R.   This view is further supported by the following
facts: that the nature of commonly observed laboratory transformations
(over long periods of time) are from the sporogenic to the asporogenic

type of culture; that it has been commonly reported an easy matter to transform a sporogenic into an asporogenic race; and that it is more exceptional and difficult to produce a spore race from a culture which, after repeated, careful microscopic examination, is seen to contain no sporebearing cells. It may be added in passing that the same circumstances hold for many species of yeasts, in which it seems probable that similar dissociative reactions occur.

In conclusion it should be said that the observation that anthrax cultures can be split into these two types, one sporeforming, the other sporeless, lends weight to some recent views (Mellon) that endospore formation may possess a significance somewhat distinct from that of a reaction to unfavorable environment as commonly assumed. Whether this phenomenon, like arthrospore formation among cocci, may be regarded as a "cover" for nuclear reorganizations, that demand physical protection, as suggested by Mellon, must be left a question. If this should prove true, however, we should come to regard asporogenic strains of sporeforming bacteria as forms which have lost some power of sexual reproduction, and which in this respect might be analogous to the asporogenic yeasts.

Dissociation and Chromogenesis.—In closing this section, one other interesting point relating to the R type cultures may be noted. This refers to the marked tendency for the appearance of a yellow or brownish chromogenesis among these forms. The fact was observed perhaps first in the case of Vibrio proteus (R variants) by Firtsch[178] in 1888. Eisenberg[150] and others have described the same phenomenon in the "dunkel" forms (probably both O and R types) of the cholera vibrio. A yellow colony form was reported by Balteanu[38] in 1926. Yellow or yellowish R types of B. diphtheriae have been described by Roux and Yersin,[416] Corbett and Phillips,[110] Bernhardt,[49] Heinemann[239] and many others. I have observed these chromogenic forms many times in the R types of the Park 8 strain of B. diphtheriae. Baerthlein,[28] many years ago, described the brownish white variant of Micrococcus citreus, presumably an R, and E. M. Brill, a student in my laboratory, recently obtained what is apparently the same form through the influence of a lytic agent which he isolated from sewage contaminated water. In this connection, I may add a curious feature relating to this culture. When growing in pure culture on agar, it forms flat, brownish white colonies; but, when growing in a mass of citreus S, it forms small, spider-shaped colonies which make distinct depressions in the mother culture. Almquist[3] has described a yellowish form of B. typhosus. In

addition to the above cases, Atkin [21] described as yellow or yellowish his type II and IV meningococcus colony variants. Novy and Soule [499] have observed an orange yellow form of B. malleus which undoubtedly represented an R type and which later gave rise to the normal, non-chromogenic culture through the formation of a regeneration fringe. Seligmann [437] in 1919 observed, in connection with a study of the cause of slimy bread in Berlin, a culture of B. mesentericus which gave off "mutations" of a distinctly yellow type and was believed to be identical with B. berolinensis.

In the case of B. proteus, yellowish colonies possessing unusual characteristics and perhaps representing R type culture, were observed by Braun and Schaeffer.[66] Fejgin [169] also has found five types of B. proteus X19 secondaries to transmissible autolysis which in colony form on agar plates were characterized as being, respectively, white, round, opaque, irregular and chromogenic. Two of the latter type were bright yellow and one was canary yellow, the pigment of all three being soluble in alcohol. In serological tests the antigenic character seems to have remained close to that of the original X19, but in two of the yellow types the agglutinability was much diminished. All were gram-positive and the bright yellow strains were found to contain long filaments along with the rod forms. The growth of the canary yellow strain only was spreading, the organisms small and nonmotile. D'Herelle [247] believed that all of these cultures were "mutations" produced by the bacteriophage. It is clear, however, that the presence of the bacteriophage is not necessary for Mr. Weaver, working in our laboratories, has produced from B. proteus apparently similar yellow strains without the use or presence of the proteus bacteriophage. Whatever the cause of this curious phenomenon may be, there is no doubt that a careful survey of the literature would yield other instances in other bacterial species.

Conclusion.—In concluding this section it may be said that the commonly observed characteristics of culture form, cell morphology, motility, possession of capsules, possession of endospores, and of yellow chromogenesis (within limits), are distinctly correlated with the bacterial type coming into prominence in the dissociative reaction. These observations should impress upon us the great danger involved in making use of such characteristics, without reservation, as a means of species differentiation. When we say that a certain organism is "motile" or is "capsulated" or is "a short rod," or "forms endospores," or "is a yellow chromogen," all that we can truthfully denote by these descriptive terms

is that the organism is motile or capsulated, or has the form of a short rod, etc., at a certain stage in its life history. It thus begins to appear that for purposes of systematic bacteriology the registration of the patent characteristics of an organism or species is by no means so simple a matter as we have been accustomed to believe. The old descriptive terms may still possess a value if we know the definite state or stage of the culture to which we refer. If we do not, these terms are meaningless.

6. FURTHER CONSIDERATION OF THE NATURE OF THE DISSOCIATIVE REACTION AND OF THE "THIRD COLONY INTERMEDIATE"

While, as has been noted earlier, it is the S and R colony types that come into greatest prominence in the majority of dissociation reactions so far observed, in other cases the situation is complicated by the appearance of at least one, and sometimes several other variants, also manifesting themselves as do both S and R by their characteristic colony form and cell morphology. Baerthlein[30] for instance in dealing with colony variation in certain bacterial species presents so many colony types that one can easily become confused and unable to recognize the actually significant forms, much less their sequence of appearance or disappearance in the original culture. The same is true of the serologic variants of B. proteus described by Felix;[172] also of the colony variants of Vibrio cholerae pictured by Eisenberg.[150] The occurrence of such a degree of variability observed in many of the early studies was unquestionably sufficient almost entirely to obscure the view that through it all existed a definite trend of cultural modification. But we are now coming to see (as indeed was strongly suggested by Gruber and his pupils[173] as early as 1888) that, in the kind and degree of variation, there exists a certain constancy and orderliness, involving not only what we recognize today as the S and R forms, but the socalled intermediate forms as well. Indeed it becomes apparent from such studies as those of Firtsch[178] (Vibrio proteus), Eisenberg[150] (Vibrio cholerae), Bernhardt[49] (B. typhosus, B. diphtheriae), Preisz (B. anthracis) and many others, that there exists a distinct parallelism in different bacterial species with reference to the appearance and behavior of the intermediate culture types and particularly with reference to the "third colony intermediate." When one has reviewed carefully the detailed descriptions of the culture changes in many bacterial species, it becomes entirely clear that the chief difficulty of interpretation in the past has been the circumstance that in some cases the S → R transformation occurs directly and sharply without intermediate steps, while in other instances there enters into the disso-

ciative reaction a transitional type. This may be represented by a single
colony form, as the third colony intermediate, which possesses character-
istics quite different from either S or R, or by a fairly closely connected
series of such intermediates, or "Zwischenformen," as they have been
commonly termed. Some of these Zwischenformen may more closely
resemble S while others are more like the R type culture. The chief of
these, however, and the one which is constant, even though the others
are lacking, is the third colony intermediate. The evidence that this
form is actually intermediate in nature will be presented shortly.

As to the probable reason why in some cases only the two chief types
of colony, R and S, can be recognized, and why in other instances we
observe either a single or a variety of intermediates, with S at one end
of the series and R (extreme variant) at the other—I believe it lies in
a differential death rate of the various cells. It was the greatest merit
of Bernhardt's study to present the first evidence for this conception
which, as we shall see, contributes to a better understanding of many
peculiar phenomena in the field of microbic dissociation. To this sub-
ject we shall return.

Sudden or Gradual Appearance of the R Type.—Among those lytic
and variational phenomena which we now include under the head of
dissociation, one of the important aspects has been the question of their
sudden or gradual manifestation. And here the reports have often
been contradictory. While Baerthlein has emphasized the sudden nature
of the S $\rightarrow$ R transformation, amounting practically to a mutation in
the usual sense, Bernhardt, working with the organisms of typhoid and
diphtheria, has equally emphasized (and, one may add, more conclu-
sively demonstrated) its gradual and progressive occurrence. I may
use as an illustration of this, however, the dissociation of the capsulated
bacteria, such as Friedländer's bacillus or M. tetragenus. In Baerth-
lein's and my own experience with the former culture the change from
a capsulated to a noncapsulated form has been quick and sharp. The
former passed directly into the latter apparently without the appearance
of transitional or intermediate forms; and this was also true of the
colony picture. Toenniessen,[462] on the other hand, emphasized the
occurrence of intermediate degrees of encapsulation which he related to a
slow process of transformation. He reported even that he was able
to "fix" the grades of encapsulation more or less permanently.
De Kruif [119] pointed out that the dissociation of Bact. lepisepticum
is very abrupt and he mentioned no intermediates; while P. B. White,[487]
on the other hand, represented the transformation of the hog cholera

bacillus as gradual, characterized by "degrees of roughness." The gradual nature of the change is also brought out forcibly by the study of variation in B. diphtheriae by Roux and Yersin [416] in 1890. Also by Braun and Schaeffer and by Friel for proteus dissociation, so far as its morphological and cultural features are concerned; and both Weil and Felix [172] in case of Proteus X19 have indicated in great detail the wide range of serologic types which lie between the H and the O forms. The same has been shown by Weil and Felix,[484] Furth,[188] Breinl and Fischer [71] for B. paratyphosus, by Breinl [69] for dysentery organisms, by Feiler and by Furth [190] for the Aertrycke bacillus and by Grushka [217] for B. enteritidis, confirming the important work of Sobernheim and Seligmann [446, 447] on B. enteritidis transformation. Felix [172] particularly has emphasized the importance of the transition forms ("Uebergängs- formen") which in B. proteus may show all degrees of serological rela- tionship between the "Ausgangsform" and the "Endform." In this respect his work is in agreement with the earlier work of Eisenberg on intermediate forms of colony variation in the cholera vibrio, as well as with the "Zwischenformen" detected (especially in colonial variation) by Bernhardt in the case of B. typhosus, B. paratyphosus, B. diphtheriae and other species.

White [487] has noted that the change from S to R in certain Salmonella cultures is sometimes abrupt and sometimes gradual. He stated (p. 72): "In some cases roughening of a culture seems to be a gradual, insidious process; in others it seems abrupt, mutative. Sometimes rough colonies picked from a plate culture show every grade of variation in their agglutinative behavior; at other times the culture is sharply divided into perfectly smooth translucent colonies and rough colonies which never flocculate." It can scarcely be doubted that some of these colonies come under the head of what we have termed the intermediate type. Several recent writers on serological subjects connected with bacterial variation have gone far astray in their conclusions as a result of their failure to comprehend these fundamental aspects of the dissociative reaction.

Postponing for the moment the earlier and more striking work of Firtsch in this field, which possesses points of special interest, we may consider the later study of Eisenberg [150] on variation in the cholera vibrio. Among the four colony variants described by Eisenberg (three of which had previously been described by Baerthlein) one ("helle") appears as the S, another ("geschwulste dunkel") is analogous to the R and the third ("Ringform") has an uncertain place. The fourth type ("Uebergängsform"), however, is of special interest in our present

consideration.   This form of colony had a size equal to, or larger than, that of the "helle" but was opaque, highly convex and had precipitous edges.   Eventually the center became depressed and an outgrowth of the "helle" form appeared from the margin, thus showing reversion. In aging cultures this "Uebergängsform" disappeared and was replaced by the "dunkel."   Regarding the frequency of appearance of the transitional type it is shown by Eisenberg's tabulation (p. 6) [150] that it may be quite absent on plates poured from cultures left to age.   The chief point for our present interest is, however, that Eisenberg gave clearly the details of the transitional trend of the S type culture through an intermediate form to the "extreme variant" R.   He thus confirmed the earlier report of Firtsch for Vibrio proteus, to be mentioned shortly. The transitional form of B. anthracis as described by Preisz,[394] involving a slimy type of culture made up of organisms capsulated in varying degrees, has already been referred to in section 5 and does not require further mention here except to state that it is manifestly analogous to the other transitional forms being considered.

For the typhoid, paratyphoid and diphtheria species Bernhardt [49] described "Zwischenformen" occurring between the normal type and the "extreme variant," that is, between S and R.   The following concerns mainly the intermediates of B. typhosus and paratyphosus.   The colonies of the intermediates, which possessed characteristics quite different from those of the S or R forms, did not arise from young, normal cultures nor from old cultures, but appeared with the apparent beginning of dissociation, increased for a certain time and then disappeared again.   Thus, at the beginning of dissociation or prior to its appearance, there were only normal (S) colonies while at the end of the reaction (quite old broth cultures) there were only the "Endformen" (R).   At a certain intermediate stage, however, one could detect all three colony types.   As will be seen presently, this circumstance of the sudden appearance and immediate disappearance of a transitional culture type is a duplication of the observations made by Firtsch at an earlier date on Vibrio proteus.

But the most curious and perhaps most significant aspect of Bernhardt's work with these transitional colony forms and correlated cell forms was the peculiar growth characteristics of certain members of the intermediate group.   This aspect of his study merits the presentation of further details, and for reasons that will later become apparent.

In B. typhosus, as in other species, Bernhardt observed not only the normal colony type (S) but also its extreme variant (R).   Along with

these, however, were several intermediate forms. Although most of these intermediate culture types grew well on gelatin as also on other mediums, there was one colony type in particular, representing one of the "Zwischenformen," which on further transfer failed to grow on either gelatin or agar at 22 C. in two days. Neufeld according to Bernhardt had already pointed out a similar phenomenon. This form of culture was apparently extremely "sensitive." There was one way, however, in which such colonies could be made to perpetuate themselves. If the dilutions from the original colony were made in broth, plating yielded no growth. But, if the dilutions were made in salt solution, colonies often appeared on the plates, gelatin or agar. This culture therefore possessed the remarkable characteristic that it was destroyed in broth but lived in salt solution. To explain this curious circumstance we must assume either that the broth was germicidal for the culture (or otherwise unfitted for promoting growth), or that it assisted the organisms to destroy themselves. In view of the lytic tendencies of the original colonies on agar the latter view seems the more probable. If this is true we have here certainly a lytic and possibly a lysogenic culture type. And at this point we may remind ourselves of the interesting work of Bordet [61] dealing (in this case with bacteriophage action) with those culture forms of the lysogenic type of B. coli which disappeared in broth although they might yield a lysogenic growth on agar if transplanted from agar.

It may be noted, furthermore, that these peculiar colony forms of Bernhardt which were incapable of further cultivation did not occur in every culture, nor were they present in young, "normal" or old cultures. Their time of appearance was rather narrowly limited. Bernhardt was of the opinion, however, that this type might be present more commonly than appeared, but that it was destroyed in the process of culturing. Bordet has employed exactly the same argument to account for the disappearance of certain cell types in the case of B. coli in relation to the lytic agent. A consideration of the possible significance of these observations for the problem of transmissible autolysis is reserved for a later page.

To the foregoing I should like to add that in certain cultures of B. diphtheriae and staphylococcus I have observed bare plaque-like areas which I have termed "invisible colonies." They occur on a background of apparently normal culture and resemble lytic areas produced by the bacteriophage. On close examination, however, there can often be detected over the surface of these spots an extremely thin film of

growth which at first does not increase.   Eventually a sprinkling of
secondary colonies may arise on such sites and it may be noted that the
picture so produced bears resemblance to lytic areas with their secondary
colonies as produced by the bacteriophage.   Similar "invisible colonies,"
barely detectable, develop occasionally in isolated form and not sur-
rounded by other culture material.   These also may present, after a
time, the phenomenon of secondary colony formation.   Microscopically
such colonies are composed of extremely small forms and usually give
no immediate growth in broth.   Transferred to slants they may yield a
delicate growth that seldom survives for more than a few transplants.
These colonies may have something in common with the nonviable forms
of Bernhardt; also with similar faint growths of slight vitality described
by Arkwright for Shiga.   They may also stand in relation to the sec-
ondary colonies (lytic stage) of Preisz, Pesch and Katzu (B. anthrax)
and of Andervont and Simon (B. cereus); also of Sonnenschien for
Monilia, as mentioned earlier in these pages.

   We may now return to the question regarding the probable reason
why the intermediate colony forms appear in some cases of dissociation
but not in others.   That one or more of them occur quite commonly
in natural dissociations is indicated by the frequency with which the
third colony type has been reported in the literature.   In the first place
it is strongly suggested by the work of Bernhardt [49] on typhosus and
paratyphosus that the possibility of revealing this intermediate or
transitional form will depend in part on the particular stage in the disso-
ciative reaction at which the colony sampling is made.   If too early the
majority of the organisms will still be in the S form; if too late the
majority will have passed over to the R.   There is a point, however,
when the transitional type may be obtained, sometimes mixed with S,
at other times mixed with R.   It is indicated by many observations,
however, that once the transition has started the tendency for its com-
pletion is strongly manifested by the culture or colony.   What lies
behind this tendency is a question of much interest.

   Although the time of sampling a dissociating culture is thus indicated
to be one controlling factor in the revelation of the "third colony inter-
mediate" there are doubtless other factors; and the nature of one of
these is also suggested by the valuable contribution of Bernhardt.   It
seems to depend, as has been earlier intimated, upon a differential death
rate or transition rate, among organisms of the S and transitional type.
There exists, as Bernhardt has shown, a stage of transition as indicated
by a certain colony form in which the organisms are not viable when

transplanted to other mediums; and this point has been confirmed by observations by Bordet for B. coli, and by Mellon [325] for B. fusiformis. These observations may possess the significance of suggesting to us that the dissociating culture is not merely a culture undergoing transformation, but a culture, certain cell components of which, are experiencing complete annihilation, at least when surrounded by a fresh growth medium. We can perhaps see added evidence of this in the acute dissociations shown by B. pyocyaneus, in the acute lysis of B. anthracis and Monilia, as already described, as well as in the socalled suicide cultures. But the point which I desire especially to make in this connection is that, in different bacterial species, there may easily exist differences in the degree of stability of this highly unstable "third colony intermediate." And this circumstance can assist in explaining many of the variable phenomena observed.

The Intermediate Forms and the Sequence of Types.—Considerable reference to the existence of the intermediate or transitional colony types lying between S and R has now been made, but without the presentation of sufficient evidence supporting the fact that they are actually transitional in character and behavior. This important point may now be considered.

Although Bernhardt presented valuable data bearing on this point with special reference to B. typhosus, B. paratyphosus and B. diphtheriae, and although similar observations have been made by others and most recently by Mellon and Enderlein, it is in the old work of Firtsch [178] in 1888 in Gruber's laboratory that we find, for those early years, the deepest recognition of the actual problem involved and thorough-going experimental attempts to throw light, not only on the cultural features of the variants themselves, but also on the trend of the transitional process. Firtsch studied the variants of Vibrio proteus (Sp. Finkler-Prior) occurring, as we should now say, under the conditions of natural dissociation in old gelatin stab cultures. In such cultures he observed the production of several variants from the normal vibrio and these he termed variants I, II and III. All could be differentiated both from the normal culture and from each other by growth in gelatin and on agar or potato, by colony formation and by the cell forms. The rapidity and sequence of their appearance depended on the time of aging of the cultures concerned. A brief review of the most interesting of his results follow.

Although gelatin previously seeded with normal culture, and sampled by plating methods, up to eight days gave only "normal"

colonies and cultures (N), beginning with the 14th day, variant I
appeared. In colonies it was brownish, glistening and thrown into
projections and folds. On agar it reverted to N growth; in gelatin stabs
it showed slower liquefaction than N. When plated on gelatin it gave
brownish colonies which kept the same form if transfers were made at
rapid intervals. If, however, the plates stood four, six or eight days
between transfers some of the succeeding colonies developed a delicate,
extending halo of growth, and transferring from these fringes gave
normal culture again. Firtsch stated that variant I was always present
in old cultures of a certain age. It showed no motility—to which fact
Firtsch attributed its different colony form.

If the gelatin stab cultures made from N, instead of standing 14
days stood 48, 73 or 94 days, Firtsch found present in such cultures
not only N and variant I but also a second type, variant II. The number
of organisms of this type increased with further aging of the culture:
at 14 days, 0; 48 days, 8% of total organisms; 73 days, 23% ; 94 days,
73%. He ascertained, moreover, that variant II could arise, not only
from N, but also from variant I if such a culture stood 56 or 67 days
or more. Variant II gave colony characters (yellow, flat, granular)
quite different from both N and variant I. It also showed a different
form of growth in gelatin.

If the standing culture was observed over still longer periods, N and
variant II survived but variant I disappeared. Thus, after 70 days in
gelatin stab:

Top layer gave—		Bottom layer gave—	
Normal colonies	208	Normal colonies	10
Variant I colonies	0	Variant I colonies	0
Variant II colonies	18	Variant II colonies	7

From these and many other similar experiments we may conclude
with Firtsch that variant I was highly unstable and was able to give
further variation either into N (slow passage on gelatin or on agar) or
into variant II (old gelatin stabs). Variant II, on the other hand, was
stable. Neither gelatin stabs, nor slow gelatin plating, nor beef broth,
nor agar, nor potato, nor either slow or rapid transfers on any medium,
nor growth at 37 or 21 C., nor transfers in series of many separate
colonies, accomplished any sign of reversion either to variant I or to the
normal form (N).

But, notwithstanding this apparent stability against reversion,
variant II ultimately did undergo further transformation into a form
which we may safely regard as the "extreme variant," the variant III

of Firtsch. A gelatin stab had been inoculated with N culture. After 375 days the colony count gave: some N, also some II, and a few III, but no variant I. After 476 days there were found, among the then greatly reduced population in the same tube, still some N forms together with some variant III, but no I or II forms. The growth of variant III on gelatin plates was very slow but ultimately gave—first, small blue-white, but later brownish, colonies with a "crumbly" surface and finely dentated margin. It was commonly nonmotile, did not grow on potato and reversion to any of the earlier types was never observed. Firtsch therefore regarded it as a permanent modification. From the foregoing exposition we can see clearly the relation of the vibrio types observed by Firtsch to our chief colony forms. His normal type (N) is the normal S; his variant I is the intermediate (O), which may generate either S or R, depending on age and circumstance; his variant II is the first R type, incompletely stabilized; his variant III is the "extreme variant," R—or, as we shall later propose, the R^n.

The splendid work of Firtsch at the time of its appearance was, as we should expect, relegated to the field of useless observations along with other similar work of the day. At the present time, however, I believe it stands as one of the most noteworthy contributions to our knowledge of the dissociation phenomenon; and, fortunately, more than a quarter of a century after its publication, when the bacteriological world had become a safer (though not too safe) place for the variationists, was made the basis of Eisenberg's comprehensive, though less critical, study of variation in the cholera vibrio.

Firtsch concluded his contribution with the following pertinent words: "In all dem heissen Kampfe, der über Monomorphismus und Pleomorphismus der Bakterien geführt wurde, bleib bisher Eines völlig unberührt, wurde Eines von allen Seiten als der 'ruhende Pol in der Erscheinungen Flucht' angesehen: die Form der Kolonien auf festen Nährboden."

Returning to the general consideration of the intermediate types and the reasons for their presence or absence in dissociating cultures, the influence of the medium may be mentioned. Since, as we have seen, slight differences in the reaction have an effect on the dissociative and lytic reactions, and an alkaline condition favors the rapid and complete transformation of type, the appearance or nonappearance of colonies of the transitional form may depend to some extent upon this circumstance. But, whatever the explanation may be, I believe that there now exists ample evidence to assure us that, standing between the S and R

types in the course of transformation, there exists a transitional form of culture which manifests itself in the "third colony intermediate" or in a closely related colony of the transitional form. This culture type, for which I have proposed the symbol, "O," is apparently both the "progeny" of the S and the "progenitor" of the R. It is such an unstable culture form (which we can picture to ourselves as ready to lean in any direction) that Mellon [326, 327] may have observed when he has spoken of the marked influence of the environment in determining the nature of the succeeding growth of certain cultures. In any case, this group of transitionals embraces certain members characterized by extreme instability and often difficult, or sometimes impossible, of further cultivation, especially in broth medium, although they are more stable in physiologic salt solution or on agar. It is probable that those cultures which afford the remarkable "fading-away" appearances, characteristic of the "suicides" and amounting in some cases to an almost complete annihilation of growth, belong to this group of transitionals. If the lytic tendency is acute, such cultures may usually be depended upon to leave amidst their lytic residues their natural transformation product, namely, the R type culture. In all this discussion, however, it should be borne in mind that these peculiar transitional forms represent a series of apparently closely related types, some of which are more stable and susceptible of perpetuation, for a time at least, on ordinary culture medium. Of this varied group, the "third colony intermediate," which has been most frequently described, is only one form.

In concluding this subject of the intermediates, a word may be added regarding the relation of these culture forms to various incitants to dissociation, a fuller consideration of which is presented in a later section. It has been demonstrated in numerous instances, including B. anthracis, B. diphtheriae, B. proteus, and members of the colon-typhoid-dysentery group, when observed in the dissociative reaction, that the first observable sign of the influence of the unfavorable condition or environment is a transition from the normal S type organism to elongated, filamentous or fungoid forms of growth, accompanied by beaded or swollen rods, large and small coccus forms and various "involution" structures often including the so-called "giant coccus" which, from the recent descriptions of numerous observers, is manifestly identical with the "Ferran'sche Körperchen" observed and pictured (Taf. VI, fig. 12) in the work of Firtsch (1888) on Vibrio proteus. It is moreover this varied assortment of bizarre cell forms

that constitute the major population of the colonies or cultures of the transitional type—a type which has for its chief characteristics a heavy and voluptuous growth accompanied by a mucous or gelatinous consistency. Such colonies are probably related to, if not identical with, the colon colonies which Mellon [331] has regarded as the mother form of B. coli mutabile. They have been described by investigators too numerous to mention. Some of the clearest references to them, however, may be found in the works of Hort, Mellon, Eisenberg, Feiler, Bernhardt, Baerthlein, Fletcher and Enderlein, not to omit the earlier study of Massini.

Conclusions.—In summarizing this section it may be pointed out that, although this review deals largely with the two chief types (S and R) involved in the dissociative reaction, microbic dissociation is not so simple a phenomenon as the "splitting" of the R type away from the normal S, as we might expect in an ordinary mutation. As we watch this reaction going on before us on plates or in culture tubes, superficially considered it seems a simple process; but we may remind ourselves that there must lie behind it all a mechanism. What this mechanism may involve, we do not yet know because we have not been able to detect or to isolate all the factors. But one thing is apparent: The R type is not a direct, but an indirect product of S; its production comes about through the functioning of an intermediate stage of culture development. Sometimes we are able to detect this transitional form by the use of appropriate cultural methods, while at other times it quite evades detection and one might thereby be led to conclude that it is absent or lacking in the transformation process. That, on the contrary, it is present in all cases is strongly suggested by several important observations which show that the transitional form (particularly in the guise of the "third colony intermediate") is a highly unstable and sensitive phase in the life of the culture. Why this is so, we do not know. But it is certain that its vitality on ordinary culture mediums, particularly in broth, is often slight. It disappears quickly from our view and leaves us—if anything —a modified culture of the R type which it seems to be at least one of its functions to produce. More than this cannot be said at present regarding the transitional type, although some of its antigenic characteristics are considered on a later page. It may eventually appear, however, that while other aspects of dissociation are given more prominence in this paper, it is the "third colony intermediate" and its nearby colony relatives among the transitional forms, that carry the greatest significance in microbic dissociation.

7. DISSOCIATION AND BIOCHEMICAL REACTIONS

Variations in the biochemical behavior of microorganisms, especially with reference to fermentative reactions, were naturally the first to be studied after morphological and cultural variations; and biochemical disparities were clearly recognized long before the study of serological and antigenic eccentricities had gained much headway. In later years, however, the increasing conviction that serologic and antigenic criteria of the constitution of bacterial species and varieties give information of a more fundamental and dependable character, has resulted in a correspondingly diminished activity in seeking out and studying fermentative or other biochemical variants. Unfortunately, morever, in most cases where clearcut serologic variants have been discovered, there has been a noticeable lack of effort to correlate these with biochemical changes in the same cultures. And the reverse also is true: that, when outstanding fermentative variants have been obtained, there has been slight attempt to correlate these with serological changes. Many of the variations in fermentation have been brought together by Gurney-Dixon.[220]

It is quite true that fermentative divergencies from the normal reaction have been numerous; but the slight degree of the departures, the apparent failure to work with pure line strains, and the lack of data bearing upon correlated differences, cultural, morphological or serological, usually leave us in doubt regarding the relation of the observed changes to the dissociative reaction as we have come to know it. This circumstance renders the majority of such contributions of slight value for the purposes of the present review. For this reason the majority of them have been omitted, and there consequently appears a lack of balance between the biochemical and serological aspects of the dissociative phenomenon. Such a discrepancy might seem to imply a certain lack of significance of the purely biochemical reactions in dissociation. I believe, however, this would be an unwarranted view of the matter. From the few cases which merit presentation by reason of their clearly observed relation to the phenomenon, it appears that the biochemical changes involved, though perhaps often less significant than the serologic, are in reality of much importance; and, when further studied, may add new and valuable facts to our knowledge of the dissociative reaction.

Dissociation and the Production of Pyocyanin.*—We can often observe that a pyocyaneus culture showing erosive action on a solid cul-

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* This illustration of a certain biochemical aspect of dissociation also enters the field of transmissible autolysis, but is none the less applicable to the case in point. The details of the reaction have been reported in full in another paper by the author.[224]

ture medium gradually loses its power to produce the blue pyocyanin and, little by little, comes to the stage of a fluorescogenic, but non-pyocyanogenic organism. What is happening to the population of such a culture? If, at varying intervals in the progress of the change, we plate out a suspension in broth, it is first ascertained that perhaps 99 out of every 100 organisms yield a rich blue-green colony. But gradually these bacteria are replaced by organisms (colonies) which produce no pyocyanin whatever. We may thus have the changing ratio: 99 : 1 ; 75 : 25 ; 50 : 50 ; 25 : 75 ; 1 : 99 ; and, in all probability, even 0 : 100. The change in this biochemical character therefore does not involve "a little loss" of pyocyanogenic power on the part of all the organisms present, but the sudden appearance of a new type of organism (the nonpyocyano-genic) and an increase in this form to the greater or less (and in some cases complete) exclusion of the "normal" blue type. I possess several such cultures which have been dissociated for over three years and none of them shows any tendency as yet to produce even a trace of pyocyanin as demonstrated by the chloroform test. It seems safe to say that where-ever pyocyanin production can be awakened in old cultures which may appear to have lost the ability to produce it, it is usually due to the regeneration of a relatively few organisms of the sensitive type which have managed to survive amidst the overwhelming population of non-pyocyanogenic forms. But, when the culture is founded on such a "mutant," or has through aging or an unfavorable medium lost the last sensitive organism, then the bluing character has, so far as I have been able to ascertain, been lost beyond power of recovery. This circumstance is opposed, however, to other studies clearly indicating the possibility or reversion of the R to the original form in other bacterial species.

Dissociation and Proteolytic Power.—Another biochemical character-istic in which the R dissociate is likely to vary from the mother type is proteolytic ability, and this has been observed in two markedly proteo-lytic species, B. proteus and B. pyocyaneus; also Firtsch [178] in 1888 pointed out the diminished gelatin-liquefying power of his variants (O and R forms) of Vibrio proteus. Braun and Schaffer [66] in 1919 reported the diminished power of gelatin liquefaction of their O forms of Proteus X19, whether produced by the influence of phenol or of starvation. Baerthlein's "second variant" of B. proteus (the O type?) is also reported to have given very slow liquefaction compared with the normal type. The same was true of Balteanu's [38] opaque variant of Vib. comma. Its coagulating action on milk was correspondingly slow.

I have already reported the same phenomenon in B. pyocyaneus, in which
the R type is inactive biochemically as compared with the S form.
Soule [450] has shown a retarded rate of gelatin liquefaction for his R
type strain of B. subtilis.

Dissociation and Fermentative Ability.—To be certain of the rela-
tion between products of dissociation and fermenting power of organ-
isms in many of the cases reported is a difficult matter. This is
occasioned by the well recognized differences not only between different
strains of the same organism, but also in the same strain at different
times in its history. To what extent these differences may be due to
dissociative reactions that are too slight to reveal themselves in physical
modifications of the culture we do not know, although it cannot be
doubted that unrecognized dissociations are occurring in perhaps all cul-
tures. It is only when the dissociates have been revealed, isolated, and
studied as separate cultures that we are justified in entertaining any con-
clusion regarding the effects of dissociation on fermentation power.
And naturally the same conditions hold for the study of virulence, sero-
logic activity, and indeed of all biochemical reactions. Up to the present
time, as has been said, there have appeared few reports which can justi-
fiably be employed for the purpose of this review, although citations of
the most diverse variability in fermentative reaction are numerous in the
literature.

Interest in modified fermentation power of variants dates largely
from 1906 and 1907 when Neisser and Massini made their first observa-
tions on the "mutations" of B. coli mutabile. Neisser's [362] observations
showed that an organism of coliform type, previously lactose-negative,
suddenly threw off colonies demonstrating lactose-fermenting power,
and retaining this ability with considerable tenacity. Massini,[320] one year
later, studied the same organisms more thoroughly and confirmed the
observations of Neisser. On lactose-fuchsin agar the culture produced
at first only white colonies but, after the second or third day, many of
the better isolated colonies showed papillae ("Knöpfe") which were
white at first but later red. One-tenth per cent of lactose was sufficient
to produce this result, but it was not produced by dextrose, mannitol or
other carbohydrates. When white colonies were subcultured within 24
hours they gave only white colonies, which produced red papillae later.
When the red papillae of the white colonies were subcultured to similar
mediums, they give both red and white colonies. When the red colonies
on these plates were further plated they gave red colonies only. These

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were fairly permanent in their new character after they had been carried for some transplants on lactose medium. The red colonies themselves never gave papillae.

These observations were carried forward by Burk,[84] by Kowalenko,[286] who succeeded in confirmation tests by Burri's single cell method; and by Reiner Müller.[357, 358] The latter obtained similar papillae-forming colonies from feces. He found that nearly all of 120 typhoid cultures gave this colony reaction on rhamnose agar while it was obtained in only a few of 200 coli cultures. The newly acquired character seemed permanent and he pointed out that the delicacy of the test compared favorably with the agglutination test as a means of diagnosing the bacterial type; also that this reaction was the most typical cultural feature of the typhoid bacillus. All Müller's work was controlled by single colony isolations performed by Burri's method. In addition Müller showed that, although cultures of B. paratyphosus B gave no daughter colonies on rhamnose or lactose agar, they were produced on raffinose agar. The daughter colonies were thus able to ferment a sugar which the mother type was unable to utilize. These new raffinose fermenters were never seen to revert to the original form.

In 1910 Jacobsen[265] isolated from a typhoid case a typhoid-like bacillus which gave a typical growth on Conradi-Drigalski medium. Secondary colonies appeared, however, which represented the typical typhoid form. Jacobsen therefore called his original culture, B. typhosus mutabile. These results were confirmed by Reiner Müller in 1911. Similar observations were made by Schröter and Gutjahr[435] on B. dysenteriae Shiga and Kruse which acquired the ability to ferment maltose and sucrose after undergoing prolonged growth in these mediums. The changes seemed to be permanent. Other contributions bearing upon this subject are those of Sobernheim and Seligmann[447] and of Thaysen[460] for a similar type. In 1910 Penfold[385] also confirmed the observations of Müller and furthermore studied the "mutations" of B. typhosus on isodulcite agar, of B. coli on lactose, of B. paratyphosus B and B. suipestifer on raffinose; also B. coli on chlor-acetic agar. He found that the acetic agar caused many intestinal bacteria to throw off "mutants" characterized by greater resistance to still greater concentrations of the same antiseptic. With reference to raffinose he showed that, while paratyphosus B formed papillae, the Aertrycke bacillus did not; and that this was the only cultural distinction between the two species. The papillae were formed only after seven to nine days and gave no acid colonies.

Burri [88] in 1910 made an unusually interesting study of a paratyphoid-like organism obtained from fermenting grass and termed Bact. imperfectum. This culture was at first unable to ferment sucrose, but when grown continuously on sucrose mediums some of the colonies gained this power. This sucrose-fermenting type Burri named Bact. perfectum. Both of these cultures Dobell [130] regarded as belonging to the paratyphoid group. The change undergone by the original culture manifestly resembles the change in B. coli mutabile in respect to lactose. As a result of plating different numbers of organisms of the original type (imperfectum) Burri concluded that all of the cells of the imperfectum type were able to mutate directly into B. perfectum. When the individuals were sufficiently separated on plates all behaved in the same manner; they became transformed. As Dobell pointed out, Dreisch has expressed this in the sense that "every individual had the same prospective potency." It may be noted in passing that Burri also observed the existence of transitional stages between imperfectum and perfectum. The ability to ferment sucrose was acquired only by gradual steps. Burri regarded it probable that the ability to ferment sucrose was latent in all the cells of the imperfectum type, probably in the form of a proferment.

Baerthlein [29] in 1912 obtained results in the study of 13 different strains of coli-like organisms from the normal intestinal tract which are not in exact conformity with the results mentioned above. All the strains showed the same reactions as observed in Massini's culture which showed colorless mother colonies containing red secondaries on Endo's agar. Baerthlein found, however, that after long cultivation on nonlactose agar there occurred a reversion to the nonlactose-fermenting type. Even after six to seven days there was a partial reversion on Conradi-Drigalski medium; that is, some white colonies appeared among the red. But Baerthlein introduced additional complicating features. He showed that both B. coli mutabile and typical coli strains on plain agar were transformed into two different types, characterized by colonial, biochemical and serological differences, also accompanied by different cell-forms. All of these types might revert to the original.

In reviewing these preceding cases, one further point is of interest. In the study of Massini [320] and others working with B. coli mutable it is clear that a form of culture unlike typical B. coli "mutated" into the typical form, capable of fermenting lactose. For this reason Massini believed that the mutating strain was a typhoid-like organism capable

of throwing off coli races more or less constantly. In the case of Müller, however, working with B. typhosus (typical) we see that this culture threw off colonies differing from the original and typical typhoid strain. The same was true of the typical typhoid of Penfold which threw off isodulcite-positive "mutants." And we observe a similar case in the daughter colonies of typical paratyphoid B produced on raffinose as described by Müller. Thus we seem to have the possibility, not only of a typical form of culture becoming transformed into the atypical, but also the reverse. It is only when these studies have been repeated and carefully checked with the cultural features and serological reactions, that we shall be able fully to understand the reactions observed. In the meantime, however, we can conclude that they deal with the problem of dissociation and involve biochemical changes of considerable significance.

One of the earlier references dealing with changes in fermentative power accompanying dissociation is found in Goodman's [200] study of variation in B. diphtheriae in 1908, mentioned subsequently in connection with virulence. Goodman made a double selection series for most acid reaction, and least acid reaction. After 36 selections he obtained from the original one strain which gave high acidity in dextrose and a second which failed to ferment dextrose at all—which in fact made it more alkaline. In both strains the ability to ferment maltose was diminished, while the ability to ferment sucrose was increased. The acid culture was virulent, the nonacid culture nonvirulent, a point confirmed by Bernhardt [49] in 1915. Here the assumed change to the R type (nonvirulent) was accompanied on the whole by a reduced fermentative power. Essentially the same point was demonstrated by Roux and Yersin [416] in 1890.

Revis [404] showed fermentation differences in his B (R) type of B. coli following dissociation through the use of brilliant green. He had shown earlier that new types produced by malachite green had lost the power of gas production. Esther Stearn [452] in 1923 reported gradual fermentation changes produced in water-borne bacteria under the influence of gentian violet and probably concerned with dissociation, although the accompanying cultural changes are not mentioned. Cultures taken from the gentian violet broth after 48 hours, 120 hours and 5 months showed transitions in communis A and B, communior A and B, aerogenes and B. acidi-lacti. At the end of 5 months all had assumed the fermentation type of B. communior A. The results of the dissociative process thus seem to have been cumulative.

In the recently reported changes in a paradysentery culture Ørskov and Larsen [513] showed that their variants, V and M gave fermentation reactions different from those of B and Bu. The latter pair fermented lactose, maltose, sucrose and xylose while the former pair did not. If we may regard the V form as equivalent to S, as shown on another page, the new forms in this case gained rather than lost in fermentation power.

A loss in the ability of B. lactici to ferment milk when the culture was grown in the presence of phenol has been shown by Schierbeck.[431] We know that in general phenol mediums are able to produce, or at least to cause the initiation of, the dissociative reaction; but, whether such occurred in the present case, is impossible to state since the cultural features of the modified phenol strain are not reported.

Certain references to the relation of dissociation to the production of pigment by bacteria are presented in section 11 under the heading of "Action of Antiseptics"; also in section 5.

In relation to the streptococci Eugenia Valentine [469] quite recently observed that the green (alpha) forms may be divided into two distinct types, X and Y, on the basis of peroxide production and methemoglobin formation in 18 to 24 hour broth cultures. The X type gave a positive peroxide and methemoglobin reaction, while the Y type, in cultures of the same age failed to yield peroxide and formed methemoglobin only after the addition of sterile broth or serum. These differences were not observed in four hour cultures. The growth activity of the two forms was also different, the X strain reaching its maximum growth point after 12 to 14 hours, while the growth of the Y strain began to fail after six hours. The X forms appeared to approach the pneumococcus in their peroxide producing and methemoglobin forming properties. Although these differences were not correlated with different colony types or with different cultural growths, serologic and fermentative differences were observed. It therefore seems probable that the dissociative reaction was involved in these cases, which also may have a bearing on the studies of Webster [481] on dissociation in Bact. lepisepticum.

Conclusion.—From the foregoing references to the biochemical changes accompanying the dissociative reaction, and from other references which are to be presented in section 11, it appears that with dissociation are concerned, not only variations in the physical aspects of the cells, but also physiological modifications of considerable significance.

Fermentation reactions in particular may undergo profound alterations accompanying the transformation of the S culture type to the R. It can scarcely be doubted that in many instances in which changes in fermentation capacity have been observed in cultures maintained in the laboratory over long periods, these are due to dissociative reactions. Of the cultural characteristics, however, which have their basis in the biochemical aspects of cell behavior, perhaps the most important is that of virulence—due, as we now have cause to believe, to specific substances such as the aggressins of Bail, the antiphagines of Tschistovitch or the virulins of Rosenow. To this subject we may now turn our attention.

8. DISSOCIATION AND VIRULENCE

One important aspect of the dissociative reaction relates to oscillations in the character of virulence in pathogenic bacterial species, present not only in the animal body but also in vitro. Although it may be unwise to picture to ourselves too far-reaching a significance in the small number of observations that merit being reported under this heading, it must now be accepted as a fact that they yield for the first time clearcut instances demonstrating the correlation of virulence with a definite form of culture—or as we shall see later, with a definite cyclostage in the genetic history of the species.

To the bacteriologist, as to the epidemiologist, the character of virulence has always been an elusive thing. Within limits, it is true, he has been able to control it; but he has seldom been able definitely to correlate it with other cultural, biochemical or serological features. Moreover, erroneous views have grown up regarding the distribution of virulence among the different cells of the culture. The most common conceptions relating to virulence are: that a highly virulent culture is one in which all the organisms equally possess virulence; that a moderately virulent culture is one in which all the organisms equally possess an intermediate grade of virulence; and that a nonvirulent culture is one in which all of the organisms are equally nonvirulent. It is quite remarkable that this view has persisted for so long a time, despite much evidence to the contrary. Although a different situation had been suggested by the early study of Chauveau and Phisalix [97] on B. anthracis, and still more clearly by the work of Bordet and Sleeswyk [64] on B. pertussis in 1910, as well as by many observations on the diphtheria bacillus and typhoid organism, de Kruif was the first to demonstrate clearly the relation of virulence to what we now recognize as microbic dissociation.

Bact. lepisepticum and Other Pasteurella Forms.—De Kruif [119] studied this subject in Bact. lepisepticum (B. cuniculicida), a highly virulent species and one of the best known members of the Pasteurella group; an organism long noted for its strongly aggressive characteristics. In this case, as de Kruif was able to point out, nothing like what we have postulated above regarding the nature of virulence really obtains. What does happen, at least in Bact. lepisepticum, is a transformation in cell type in which the number of fully virulent bacteria (de Kruif's D type) gradually decreases, while organisms of a new type of nonvirulent organisms (G) make their appearance and gradually increase in the bacterial population. In other words, lessened virulence in this case does not mean a similar fractional loss by each and every organism in the culture but a dilution of the number of virulent bacteria (S) by an increasing number of bacteria possessing no virulence whatever, or at least a markedly diminished virulence (R). The new type, appearing under the conditions which usually produce attenuation, is the nonvirulent "mutant." In the actual comparative virulence tests, as first reported, although massive doses of his G type (R) killed rabbits, doses of one cc. or less usually produced no lethal effect. In the rabbits that succumbed it is important that the same G type was obtained from necropsy. On the other hand, type D cultures were often fatal in doses of 10^{-7} cc. and usually in doses of 10^{-5} cc. for 1500 gm. rabbits.

Although not successful in earlier tests, de Kruif [121] reported later (1922) that relatively large amounts of type R culture were fatal to young rabbits of 600 to 700 grams. He also demonstrated that the virulence of R could be raised by passage through young rabbits to such an extent that 10^{-4} cc. might be fatal for a 750 gram animal. In some of these cases a few type S organisms were found at necropsy in the peritoneal fluid or heart blood. It is of special interest that, notwithstanding the increase in virulence, the other characteristics of the culture remained of the R type. De Kruif therefore concluded that the granular form of growth and lack of virulence are not invariably concomitant. The R cultures possessing the raised virulence were not, however, tested on adult rabbits.

To the above exposition of de Kruif's important work it should be added that the dissociative reaction in Bact. lepisepticum had been recognized, although not fully studied, by Bernhardt [49] in 1915. Bernhardt reported the production of two forms of colony, mutant from the normal type of culture. One was his socalled "geript" form; the other his "durchschleimig" type. The latter was more virulent for rabbits.

While the description of these two types is very incomplete it seems probable that the "durchschleimig" type was not the virulent D (S) form of de Kruif, but the intermediate or "Zwischenform" of many writers which is referred to in greater detail in section 6 of this paper. Although de Kruif did not report this intermediate there can be little doubt but that it is present at certain stages of the dissociative process of Bact. lepisepticum. It may be added that also in the closely related form of Pasteurella, the B. avisepticus of fowl cholera, Bernhardt recognized several colony types among which we can detect the R and S. These, however, were not studied by him in detail.

B. avisepticus (Pasteurella avium): Interesting data on the dissociation of B. avisepticus are, however, available from the work of Manniger,[317] published in 1919. It is well known that this organism seems to survive in broth cultures better than on agar slants. Manniger undertook to study this peculiarity and at intervals for one and one-half years prepared agar plates from an old broth culture. Among the colonies that appeared more or less normal he observed some that were dark brown in color and showed other irregularities. These he believed at first were contaminations, but, on further study, they were found to be mutants from the original stock. The fact of special interest, however, is that they were nonvirulent, while cultures in broth from normal colonies were highly virulent. The nonvirulent organisms were larger and plumper and possessed no capsule, while those of the virulent type were minute and well capsulated. Of the nonvirulent culture, rabbits and hens easily tolerated one-half of an agar slant culture. A whole agar slant sometimes killed pigeons. When the culture was recovered from them and then injected into mice, 0.000,01 oese was usually fatal in two days. Thus the loss of virulence was not permanent when large amounts were injected. Obviously, such large amounts of the mutant culture still contained some of the organisms of the virulent S type. These propagated in the body of the injected animal while the non-virulent mutant organisms were destroyed. A study of the relative phagocytability of the two types showed that, while the virulent organisms were not phagocytable, the mutant organisms were quickly taken up by the white cells. Manniger presents other interesting data on the immunizing value of the mutant type; also on the serologic relationship between the virulent and the nonvirulent types of culture. These subjects are presented in detail in sections 10 and 9, respectively, of the present work. It can scarcely be doubted that the two Pasteurella

forms with which Manniger and de Kruif worked were closely related; and that the former investigator was concerned with the S (virulent) and the R (nonvirulent) forms of the culture which de Kruif was able to recognize more clearly. It results, however, that each of these studies clearly confirms the other with respect to the unequal distribution of virulence among the various colonies of the respective original cultures.

Bacillus pestis: Gotschlich [204, 205] has reported dissociation phenomena in connection with his studies on plague in Alexandria in 1899-1900, thus involving another member of the Pasteurella or hemorrhagic septicemia group, B. pestis. From human buboes and also from cat pest he obtained, in addition to organisms of the normal type, a variety which, as he states, would not be regarded as B. pestis if one did not know the source. The growth on agar was characterized by heavy, shining colonies which did not grow on gelatin at 15 C. These cultures, as opposed to the normal, possessed no virulence for rats, guinea-pigs or rabbits, the latter easily tolerating 10 agar slant cultures at a single inoculation. The variant cultures showed diminished agglutinability and yielded an immune serum which agglutinated the normal culture only slightly. After some weeks in the icebox these atypical cultures reverted to the normal form and resumed virulence. It seems probable that Gotschlich dealt with the normal S type of B. pestis and, not with the R variant, but with the intermediate, O.

Probably the forms of the pest bacillus reported by Dudtschenko [137] in 1915 represented similar types. It may be added that Klein [279] in 1909 had, according to Gotschlich,[205] also called attention to two regularly occurring forms of the plague organism, one from the active human infection, characterized by possessing cylindrical rods and high virulence; the other form more common in rat pest and characterized by short, ovoid rods of greatly diminished virulence. Shibayama [439] in 1904 had also noted in plague cultures certain forms showing different cultural features and significant discrepancies in agglutination reactions, making diagnosis difficult. From these records it is thus apparent that dissociation is not limited to the rabbit and avian type of Pasteurella, but is also present in the human form (B. pestis), although here the matter has received little attention. In this disease interesting results might follow attempted immunization with the R type cultures as de Kruif has studied the matter in Bact. lepisepticum, and as I have observed the immunological results in the related organism, B. avisepticus.[223]

B. anthracis.—Although, as has been said, the studies of de Kruif have given us one of the clearest pictures of the close association between virulence and distinctly recognized type S cultures, the conception of virulent and avirulent organisms existing side by side in the same culture was not entirely new. In 1895 Chauveau and Phisalix [97] obtained from lymph glands, but not from heart blood, of cattle that had died of anthrax infection an atypical and nonvirulent organism which, according to our present view, must have been the S type.* This new culture apparently bred true. In 1904 Preisz,[393] and later Baerthlein [28] and Eisenberg,[149] recognized two distinct colony types. The first was large, transparent, somewhat indistinct and seemed to represent the normal medusa form. The latter was smaller, succulent and cloudy white, with a more glistening luster. The latter was undoubtedly transitional, as this form was made clear later by Preisz. In 1912 Markoff [319] obtained five colony variants all of which bred true for 10 to 30 generations. Among these we can recognize, not only the S and R forms, but also the more or less succulent and often slimy transitional or intermediate type (O).

In 1911 Preisz [394] showed more conclusively than in his earlier publications on anthrax the relation of colony form to capsule formation and virulence. Since this study has been mentioned in some detail under the heading of capsules in section 7, a brief statement here will suffice. When Preisz plated on agar Pasteur's first and second vaccines, he noted the appearance of three sorts of colony. The first was much like the normal medusa type and was virulent for mice. It possessed the ability to generate capsules in the body of the animals. The second colony form was a succulent and slimy type in which many of the organisms were poorly capsulated. When injected into mice death usually resulted and normal anthrax organisms (and colonies) were obtained from the necropsy material. The third type of colony differed markedly from the other two. It was round, white and fairly compact. The single organisms showed no capsules and the culture was nonvirulent upon inoculation. When the animals were killed later no bacteria of any sort could be found in the blood or tissues. This noncapsulated and nonvirulent form bred true. In the light of other dissociation phenomena we should be inclined to call the first type mentioned above the R form; the second the transitional and the last the S. Such an interpretation corresponds with the results obtained by Soule [450]

* We shall see in the following exposition that there are certain discrepancies in the apparent relation between virulence and type in anthrax. The situation cannot be made fully clear until much further study has been conducted on this problem. The statements which follow represent merely tentative conclusions. Serological tests must eventually decide the matter.

on the dissociation of B. subtilis. But, as we shall see, there are various complicating factors in the dissociative reaction as related to the anthrax bacillus.

Regarding the correlation of type and virulence Bail and Flaumenhaft [34] in 1917 described what I have termed the R and S colony forms, pointing out that the one which we regard as S was the variant from the normal (medusa) type. In a limited number of virulence tests the R form of culture showed the greater virulence for guinea-pigs. In cultures both types bred true, but in the animal body a transformation occurred from S to R. In this work it is of interest to note that in the necropsy of the pigs that had received the S culture $\bar{a}$ type of Bail the culture form varied with the location in the tissue. From the edema came only R (β type), from the heart blood mainly S, while in the spleen and liver the numbers of the two forms were about equal.

Again in 1920 Gerhard Wagner [476] obtained from human sources two colony types of B. anthracis which he termed the typical A and the atypical B. The atypical differed from the normal in being smaller (although denser and heavier), "cloudier" and more of a grayish white. The individual organisms were shorter and plumper. In gelatin the culture grew in short, thick processes, not in fine threads (inverted fir tree growth) as the typical form, confirming the earlier observation by Bail. The virulence of both for pigs seemed to be the same, but for white mice the atypical form was the more virulent. The reversion of the atypical to the typical occurred after several years. After seven years the two forms could no longer be differentiated. Excellent photographs of these types accompany Wagner's paper. They show clearly the usual characteristics of the R and S types and strongly resemble the R and S cultures recently obtained by Soule in his dissociation of B. subtilis.

The latest contribution to the study of the relation of dissociation to virulence in anthrax, although having another purpose in view, is the brief report of Gratia [212] in 1924. Gratia also noted the two chief types of colony (R and S) so often described before. One (A) was convex with regular margins and gave a homogeneous clouding in broth. Microscopically it showed short, well isolated organisms forming only short chains. The other type of colony (B) was flat, more translucent and filamentous. Microscopically it contained long chains and filaments made up of elongated bacilli. In broth it gave an agglutinative form of growth. When the A type was inoculated into nine rabbits intravenously four

survived while the others died after a prolonged period. When the B type was injected similarly into twelve rabbits eleven of them died in two to four days. When freshly isolated and then left to themselves these two culture types were reversible; but after longer cultivation each became more stable. These findings compare favorably with the results of Priesz and of Bail and Flaumenhaft, mentioned earlier.

Mr. Nungester in our laboratory has ascertained, from a long series of inoculations into guinea-pigs of pure R and S cultures, that the average time until death of the R inoculated animals was 49.5 hours while the time for the S inoculated animals was 78.3 hours. This difference was not observed in inoculated mice. The assumed transitional type, characterized by slimy growth on agar, we might conclude from the tests of Preisz and others, holds an intermediate degree of virulence. As a matter of fact Mr. Nungester found that a short series of guinea-pigs inoculated with his slimy culture type gave an average time until death of 58 hours, intermediate between the times for R and S. Of the other four culture types obtained by Mr. Nungester, all of which remained constant for 20 or more passages on slanted agar, the virulence has not yet been satisfactorily tested.

These results, suggesting a greater virulence for the R type of B. anthracis than for the S form, are opposed to the results of Wagner. With the exception of the last observer there is general agreement among the workers cited that, what we are inclined to call the R form of anthrax culture, is the more virulent, while the noncapsulated S form, giving the compact, white colonies, is nonvirulent. The transitional type, characterized by slimy growth on agar manifestly holds an intermediate position so far as virulence is concerned. So far as the R and S types are concerned, this interpretation is opposed to the findings of de Kruif for Bact. lepisepticum; and, as we shall see as we proceed with this review, it is opposed to the majority of reports dealing with other pathogenic bacterial species, where it is the S form that carries the virulence. According to Enderlein [160] the virulent stage of B. anthracis is the "Phytascit." It is also of interest to note that, if our present conception of these types should hold true, it would be the R type of anthrax, rather than the S, which has become established through many years as the "typical" anthrax culture. The final interpretation of the results already presented can be accomplished only when the whole subject is submitted to a more thorough-going study than it has yet received; and particularly we must await an answer to the question as to

which form of the culture carries the double (S-O) antigenic load; for, so far as our present knowledge is concerned, this is the criterion of last resort.

B. diphtheriae.—In the case of B. diphtheriae the question of relationship between the Klebs-Loeffler bacillus (clinical type) and the Hofmann types has been a subject of perennial interest and one on which a vast amount of inexact investigation has been conducted. While Loeffler himself adhered to the view of two distinct species and converted many to his view, other early workers, particularly Roux and Yersin [416] (1890), maintained that all the variable forms belonged to a single species and that transmutation between virulent and nonvirulent types was common. Although acknowledging the similarities in many respects, Roux and Yersin were among the first to point out distinct differences between the types, the more significant perhaps since their experiments were in accord with a belief in a species unity. These differences lay in the virulence and morphology of the organisms, rapidity of growth in broth, density of growth and acid production. They thus recognized the avirulent type as shorter, plumper and less evenly stained, giving quicker and stronger clouding, and alkaline reaction rather than acid in sugar broth. Starting with a pure line culture of the virulent type they showed clearly the origin of nonvirulent forms; also the influence of growing cultures at 39 to 40 C on this transformation. They did not, however, associate virulence with form of colony, cultural features on agar or, naturally, with serologic reactions.

More convincing experiments were reported in 1897 by a small group of workers, Hewlett and Knight, Zupnik, De Martini and particularly Corbett and Phillips in England. Corbett and Phillips [110] showed the splitting of a pure line culture to yield two sorts of colony —one thin, gray, "inconspicuous," acid-producing and highly virulent; the other whitish or yellowish white, heavier, more opaque, nonacid-forming and nonvirulent. These characteristics may easily be recognized as representing respectively the cultural features of the virulent Klebs-Loeffler bacillus and the pseudodiphtheria bacillus. I have studied the strain, "Park 8," in this connection and have found that it dissociates readily, especially on agar and under the influence of ascitic fluid, into the forms described by Corbett and Phillips and into a distinctly coccoid type.

In 1897 the unity of the diphtheria bacillus type was questioned by Zupnik.[498] He found cultures from collections, as well as from the throat, which showed on plating two different growth forms. One gave

on agar a relatively heavy, flat, dull colony with irregular borders; the organisms were gram-positive and virulent for guinea-pigs. In beef tea the growth was at the surface and with no clouding. The other type gave smaller colonies which were quite round, convex and glistening. The organisms showed Babes-Ernst corpuscles but did not stain well with Gram. Injected into guinea-pigs subcutaneously they produced infiltration and necrosis but never death. The beef tea cultures showed at first diffuse clouding but later pellicle formation and clearing.

Hewlett and Knight[251] (1897) observed that pseudodiphtheria organisms often made their appearance in normal virulent cultures and reported the change of virulent and typical Klebs-Loeffler forms into pseudo types by 17 hours exposure at 45 C. The resulting organisms were short and plump, without granules, and nonvirulent for guinea-pigs. They also transformed typical pseudo forms into virulent by guinea-pig passage and by passage through a serum medium. This has been often reported since. In the same year de Martini[125] stated that the pseudo form would grow in immune serum while the true Klebs-Loeffler type would not. This is an important observation, but so far as I am aware it has never been confirmed. Additional data favoring the view of transformation in culture have been reported by myself[221] (1907).

The most comprehensive early picture of variability in the diphtheria bacillus, however, appears in the study of Slawyk and Manicatide[443] in 1898. These authors studied in great detail the behavior of 30 strains. Anyone with patience to review the mass of data embodied in the 42 pages of tables accompanying the article can obtain a clear picture of Klebs-Loeffler variability. The same two colony types appeared: one, transparent, flat, dull; the other, opaque, convex, glistening. Between these were many intermediates. On the whole the former were more virulent for guinea-pigs, but this subject receives slight consideration.

Gorham,[202] on the basis of cultural examinations, came to the conclusion that there occurred in the throats of convalescents a transformation from granular and virulent diphtheria bacilli to solid-staining nonvirulent forms. He interpreted this change as due to the influence of body fluids of the immune individuals. This view, although unsupported by direct evidence at the time, is now amply justified in the light of more recent studies of the effect of immune serum and body fluids on the morphologic type and pathogenic characteristics of the diphtheria bacillus, as well as many other microorganisms.

Lesieur [302] in 1903 found that passage through three rabbits changed
Hoffman-Wellenhof types to the morphology of the true Klebs-Loeffler,
while cultivation in diffuse daylight for eight months changed typical
clinical forms to strains indistinguishable from pseudodiphtheriae.
Denny [124] in the same year showed that virulent throat strains changed to
pseudo forms. Others have maintained equally the invariability of the
pseudo type, and Clark [100] has alleged the separate nature of the Hof-
mann forms.

A further interesting and convincing contribution to the dissociation
of the diphtheria bacillus with respect to virulence is that of Goodman [200]
in 1908. Goodman made the attempt to split a pure line culture of a
virulent strain by means of repeated selections for high and low acidity
produced in sugar mediums. Thirty-six selections were made in series and
thus two bacterial types were obtained, one of which gave a strong
fermentation of glucose while the second not only failed to ferment but
gave an alkaline reaction. The acid forming culture was the more viru-
lent for guinea-pigs. We shall note presently that this correlation
between acid production and virulence and the correlation between alkali
production and nonvirulence was further confirmed by Bernhardt [49] in
1915, as also by many other observers. Unfortunately Goodman did
not present data dealing with the correlated morphological features of
his two culture types; but with the work of Corbett and Phillips and
others in mind there can be no doubt that Goodman succeeded in effect-
ing the dissociation of his original form, at least to the stage of an inter-
mediate, through resort to selections for high and low acidity. It is of
interest to note in this connection, however, that according to Crowell [114]
Meader in 1919 was unable to observe correlation between acid pro-
duction and virulence. Of course it must be taken into consideration in
these studies that, as Bernhardt has pointed out, there may exist pseudo-
diphtheria forms which are not true R types (diphtheroids) but which
may produce acid without at the same time being virulent. In this cir-
cumstance we have an analogy with many other bacterial species.

In a highly comprehensive study of variability in pathogenic
organisms George Bernhardt [49] in 1915 presented a mass of valuable
data including transformations in B. diphtheriae as well as in some other
species. One essential aim of the work was to demonstrate that the
transformations mentioned were not, as believed by Baerthlein, sudden
changes (saltations), but that they were produced gradually and pro-
gressively through a considerable range of transitional colony types
lying between the original culture and the extreme variant (R). Here

he established the basis (as Firtsch had done for Sp. Finkler-Prior and as Eisenberg did for the cholera vibrio) for the "Zwischenformen" referred to later by Weil, Felix and others in discussing the transitional types of B. proteus and other bacterial species. Bernhardt plated out on agar a culture of virulent B. diphtheriae coming from an old broth tube. After a week or more he obtained two colony variants: type I, large, thick, whitish colonies containing ordinary diphtheria rods accompanied by involution forms; type II, small, transparent and delicate colonies with indented margins containing small thick bacilli, often with pointed ends, and among these some club shaped or gourd shaped "degeneration forms." These differences disappeared, however, when the cultures were returned to Loeffler's blood serum. The type I cultures were highly virulent while type II was only slightly virulent in young broth cultures. In seven to ten day old broth cultures, however, there was no difference in toxicity. Bernhardt next attempted to obtain by selection an "extreme form" of type II which should have lost all toxicity and virulence. He selected type II colonies which showed no secondaries (as most of them did) and after plating further secured two types of transparent colony (type II, a and b). Type II b yielded organisms which were nontoxic and nonvirulent. They were Neisser-negative and showed polar staining. They possessed the characteristic morphology and staining properties of the Hofmann-Wellenhof pseudo-diphtheria type and were constant in further propagation. Bernhardt was also able so to dissociate a highly virulent "American strain." He could, moreover, observe the secondary type of culture in nasal diphtheria and in urine. He succeeded, furthermore, in inducing the type I (S) → type II b (R) transformation under the influence of immune serum. By animal passage he changed the S form to an intermediate type ("Zwischenform") possessing reduced virulence, even if not entirely to the nonvirulent (extreme) R form. In view of these results Bernhardt concluded that the true diphtheroids (Hofmann types) are the mutation product of the clinical diphtheria type of bacillus, but that there are also "pseudodiphtheria" bacilli which are not necessarily related to the causative agent of diphtheria.

In 1917 Heinemann [239] made an interesting observation on the relation of toxin production to morphologic type and certain cultural features of the Park 8 strain of B. diphtheriae. His results as we can clearly observe are interpretable on the data supplied earlier by Bernhardt, as just reported. The incentive to Heinemann's brief study was the circumstance that in his laboratory variations appeared in toxin produc-

tion, intermittently and apparently without cause. Film formation always occurred and no contaminating organisms could be detected. Heinemann observed, however, that the nature of the film varied. Sometimes it was thick, rough and yellowish, occasionally brownish in patches; and under these conditions toxin formation was low. Again it was more delicate, friable and light gray in color; and in this case the toxin was potent. It is well known that it is possible for those experienced to predict with considerable accuracy the amount of toxin forming by observing the nature of the film. In films of these two types Heinemann observed marked differences in the morphology of the cells. In the nontoxic cultures coccus and coccoid forms had largely taken the place of the rods. These cocci "did not appear as well rounded forms resembling typical staphylococci, but might easily be mistaken for streptococci in diplococcus form or in short chains." Intermediate forms were also observed, which "still retained the outline of a bacillus that seemed to be swollen, then broken up into cocci of various shapes and sizes." It was proved that these coccus forms were really variations of the original bacillary form. On Loeffler's (horse) blood serum typical bacilli appeared, but club and granular forms were always among them. When such a growth was transferred to veal-glucose-agar the coccus forms appeared. Transferring back to Loeffler's medium caused a return of the bacillary forms in 24 hours. Heinemann recognized the similarity between these results and those reported by Mellon [326], [327] for diphtheroid bacilli. I have seen these same changes occurring in the Park 8 strain and there can be no doubt that they involve the dissociation phenomenon. Successful plating on favorable mediums reveals the two colony types together with "intermediates," as first shown by Corbett and Phillips [110] in England thirty years ago, and by many others since that time.

The most recent study involving dissociative aspects in B. diphtheriae is that of Crowell [114] in 1925. From his original "single cell" culture Crowell was able to obtain both toxic and nontoxic strains. The toxic cultures, on further plating, gave rise to both toxic and nontoxic forms; while the nontoxic cultures produced only nontoxic. These, Crowell believed, were permanent in their lost heritage of toxicity. Unfortunately we find in this work slight record of the differential cultural, colonial or morphological features of the toxic and nontoxic strains. It can scarcely be doubted, however, in the light of earlier studies, that Crowell was dealing with the S and R dissociates.

In concluding this consideration of the dissociative reaction of the diphtheria bacillus, it seems to me that there can be little doubt that the Hofmann type represents the R form of the clinical diphtheria type; or, we might better say, represents one of the R forms. The actual position of the coccoid type which was referred to by Bernhardt as the "extreme variant," which has been described by Mellon and by Heinemann, and which I have seen in several dissociations of the Park 8 strain of the diphtheria bacillus, is still a question. At present I am inclined to agree with Bernhardt that it represents the "extreme variant" R. In any case, the remarkable persistence with which the diphtheroid forms hold to their type helps to explain the long conflict of opinion regarding the transmutation of one form to the other. At the same time, I believe that we should hold ourselves open to the view that there may exist in nature culturally and morphologically typical diphtherial organisms that either never possessed virulence or have become nonvirulent without at the same time having passed over to the R form. For such organisms it seems desirable that "pseudodiphtheria bacilli" should be used rather than "diphtheroids." It may be noted, in addition, that these pseudo cultures also may have their correlated R types, which might well be termed "pseudo-diphtheroids." According to these views, while the pseudodiphtheria culture may be differentiated from the clinical type on the grounds of virulence, the pseudodiphtheroid may be differentiated from the true diphtheroid on the ground that the latter only has the power of reversion into the virulent, clinical type. To put the matter in another light—the clinical type possesses actual virulence, while the diphtheroid carries potential virulence. On the other hand, neither the pseudodiphtheria type nor the pseudodiphtheroid possesses either actual or potential virulence. From this point of view the great mass of socalled Hofmann types comprise two distinct forms: the true diphtheroids which are potentially virulent, and the pseudodiphtheroids which are neither actually nor potentially virulent. At the present stage of our inquiry I know of no means of differentiating between these two types except by attempting to enforce a reversion. In such attempts it may be that the observation by Soule [450] in the case of B. subtilis (that a serum immune to the R form of culture has the power to effect a reversion of R to S) may play an important part. Whether the S form of the nonvirulent, pseudodiphtheroid can, under any conditions, ever acquire virulence, or whether it ever possessed any, must still remain a question; but both seem doubtful.

One further point, and one of considerable interest, attaching to these studies on the dissociation of the diphtheria bacillus is the circumstance that, not only the factors for virulence but also the factors for toxin production, seem definitely to be correlated with the S form of culture. How close this correlation actually is, remains for further study to demonstrate. But we shall observe presently that the same fact holds true for the dysentery bacillus, for B. enteritidis, and it may be in the case of B. botulinus and other toxic anaerobes.*

B. botulinus.—In further reference to the possible relation between dissociation and toxin production, as just illustrated by B. diphtheriae, the situation in B. botulinus is of some interest, although the actual facts still remain to be ascertained. The significant points to which I wish to call attention have been introduced by McIntosh and Fildes [322] in 1917, and by Shippen [441] in 1919; but particularly by Reddish [402] in Rettger's laboratory in 1921. McIntosh and Fildes pointed out that the deep colonies obtained from plating B. botulinus were always likely to yield impure cultures and emphasized the point that it is only by the appearance of surface colonies that the purity of anaerobes can be tested. They obtained from Kral's laboratory a culture of B. botulinus which they alleged was contaminated with B. sporogenes. Shippen found that some of his botulinus cultures were atypical and nontoxic. Reddish subsequently examined 19 supposedly pure cultures of B. botulinus from various laboratories in the United States and reported that 18 of these were "contaminated" with an organism giving the cultural and fermentative characteristics of B. sporogenes. He believed the other culture also would have shown the same impurity if he had made a second examination. While the "normal" botulinus colonies were smooth and regular, and gave rise to toxic cultures, the contaminant colonies were like typical B. sporogenes; that is, more diffuse, translucent and with irregular or fimbriate margins, as described in the textbooks. These last were nontoxic. The toxic cultures fermented lactose and sucrose, in addition to glucose, maltose and levulose; while the nontoxic cultures fermented the last three only. Both types of cultures gave putrefactive odors and decomposed egg-meat medium, but the nontoxic more extensively and rapidly than the toxic. Here it is perhaps of importance to note that neither van Ermengen himself, nor his followers among the

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* Our consideration of the dissociative aspects of B. diphtheriae would not be complete without reference to the work of Enderlein.[160] This author has seen in the cycle of development of the Klebs-Loeffler bacillus many cyclostages ("Cyclostadien") through which the culture passes in its life history. Of these it is the highest stage in the cycle (the "Kulminante"), marked by the socalled "Cystascit" phase, which he believes carries the supreme virulence. Further consideration of Enderlein's work is presented on page 280.

European observers, have ever described the putrefactive odor that characterizes the botulinus strains of American origin, as isolated and described by American workers. Reddish also reported that "the surface colonies which answered the description of typical colonies of B. botulinus contained, besides the botulinus organism, at least a small number of B. sporogenes." He remarks further, "On the other hand, some of the typical B. sporogenes colonies were proved to enclose B. botulinus or spores." He thus apparently demonstrated that, in the majority of cases at least, his toxic strains, coming from isolated colonies, contained both botulinus and sporogenes, while he states elsewhere that his nontoxic strains usually contained sporogenes only.

Reddish concluded that, in view of these results, no toxic strains of B. botulinus should be considered as pure cultures. His explanation of this curious phenomenon (which seems to discredit common methods of pure culture isolation by plating and colony selection), was that, "when B. botulinus and B. sporogenes are present in the same material, they are so closely associated that it is impossible to separate them." He concluded, morever, that the reason why American workers thus deal exclusively with "contaminated" botulinus cultures is because they are forced to sample, in the main, "spoiled products," while van Ermengen and his followers in Europe usually obtained their cultures from fairly clean ham.

It seems to me more probable, however, that the experimental data presented by McIntosh and Fildes, Shippen, Dickson [127] and others offer a quite different and fairly clear explanation of this somewhat remarkable phenomenon: B. botulinus of the normal S type, like the clinical type of the diphtheria bacillus, is toxic; but it readily dissociates into the R form which is nontoxic and gives a colonial, cultural and biochemical picture which (to express the point most conservatively) approximates that of B. sporogenes. The reason why the R type of B. botulinus was the invariable contaminant of the cultures studied by Reddish and other American workers, while it is apparently seldom observed in European cultures, may depend on the circumstance that the exclusively American method of isolating B. botulinus involves heating the culture before dilution and plating are carried out, a course which, so far as I am aware, is seldom followed in European laboratories. We shall note in a later section the rôle of heat in enforcing the dissociative reaction. The proof or disproof of the hypothesis presented above may afford an interesting problem for those interested in botulinus research, and may have a bear-

ing on the biology of B. tetanus and other anaerobes. The early work
of Schattenfroh and Grassberger [427] in 1900, dealing with another
anaerobe, the butyric acid bacillus, has established the lead into this field,
but as yet I believe they have had no followers, although the work of Ida
Bennington [47] in 1922 on toxic and nontoxic strains of an anaerobic
organism isolated from larvae of the green fly may bear closely upon this
problem.

To the above instance it may be added that a circumstance which
may possess similar significance has recently been noted by Hall [230] in
connection with the examination of a considerable number of cultures
of various species of spore-forming anaerobes. Hall observed that the
majority of these cultures were "contaminated," and chiefly with B.
sporogenes. In some cultures this organism only was present, and the
only pure culture was one labelled "B. sporogenes." Of course we know
that contaminations often creep into cultures of spore forming anaerobes,
or may have been there from the beginning. At the same time we know
that spore forming organisms (both aerobic and anaerobic) possess the
ability of dissociating into a form so unlike the original, in colony struc-
ture, cultural growth and biochemical and serologic reaction, that it may
readily be mistaken for a contamination by an inexperienced observer
who has not made a special study of the R types of the cultures con-
cerned. In the case of Hall, as in the case of Reddish mentioned earlier,
it might be interesting to ascertain to what extent, if at all, Hall's "con-
taminations" are merely the dissociated R forms of the original S cul-
tures, perhaps, as is so often the case, manifesting the phenomenon of
bacterial convergence, which in this instance may have brought them
together into a culture type bearing the characteristics of B. sporogenes.
In this case reference to the results of Esther Stearn,[452] involving appar-
ent convergence of certain members of the coli group might be in order;
also to the work of Grassberger as mentioned above.

In concluding the relation of dissociation to toxicity of various forms
of culture, reference may be made to the results obtained by Bronislawa
Fejgin [168] in 1923 on the Shiga dysentery bacillus. From normal Shiga
cultures she obtained three strains that were resistant to the action of the
lytic principle and possessed other divergent physiological characteristics.
While normal cultures on solid media died after a few weeks, these forms
lived several months. While the normal culture was toxic for rabbits,
the modified type, inoculated in amounts of four to five cc., gave no
characteristic lesions. These new forms grew in broth as a sediment,

gave different fermentative reactions and manifested altered agglutinative reactions. They undoubtedly represented R types.

Colon-typhoid-dysentery Group.—Although Babes [25] in 1890 had pointed out the variability of B. typhosus in colony form and certain biochemical features, Steinhardt [453] in 1904 showed much more conclusively the existence of the two chief colony types of the typhoid bacillus and correlated them with their respective forms of growth in broth. She also reported the slight virulence for animals of the spontaneously agglutinating cultures (R) compared with those cultures which clouded homogeneously (S). In 1921 Arkwright [16] first recognized clearly the S and R forms of B. dysenteriae and some other organisms, and showed a somewhat greater virulence for the S cultures. His results were confirmatory of the limited evidence pointing to the existence of two cultural forms and the greater virulence of one of them as presented by Steinhardt in 1904. In 1918 Baerthlein [30] laid the basis for the recognition of dissociation in the paratyphoids and B. enteritidis, but the virulence of the S and the nonvirulence of the R form were not made clear until the work of Topley and Ayrton [464] appeared in 1924. Jordan [273] in 1926 reported virulence for the S form of B. paratyphosus B and its disappearance in the R culture. When the reversion of the R type to S was obtained, after many generations, the virulence reappeared. Orcutt [376] in 1923 indicated the same relation between type and virulence in B. cholerae-suis; and this was confirmed in a complete and thorough-going study by White [487] in 1925 on the same species. Similar results may have been obtained by Baerthlein [27] in 1912. Gratia's [211] results on B. coli, indicating less virulence for the S form and greater for the R, are opposed to the usual findings for members of this group, but seem to be in harmony with findings in B. anthracis. We observe also in Gratia's study that it was the R form that was most actively motile, while the S form was nonmotile. In both instances these results differ from those observed by most other investigators and are in need of confirmation. In B. enteritidis Gärtner the relation of virulence to definitely recognized S and R forms has been revealed most clearly by Goyle.[206] When the respective cultures were tested on mice he found that the S form was highly virulent while the R form was notably attenuated. Of 17 mice inoculated with the S type culture all died, while from a group of 14 inoculated with the R type culture only six died; and in these the period was longer than in any of the S inoculated mice. Goyle also studied the subject of the relation of toxicity to the

two culture types and was able to show that, as in the case of B. diph-
theriae and B. dysenteriae, the filtrates of the type S cultures possessed
greater toxic power; and this appeared whether the filtrates were or were
not heated at 100 C.

B. pertussis.—There should also be mentioned here the early work of
Bordet and Sleeswyk [64] who presented data on B. pertussis, alluded to
on a future page with reference to serological reactions. It can scarcely
be doubted that their MS culture grown on a blood medium was the S
type while their MG culture grown on agar without blood represented
the R. The cultural details are not complete but they report the MS
culture as more "toxic" than MG for rabbits that were undergoing
immunization.

Capsulated Bacteria.—The correlation between virulence and the
type of culture is of special interest in connection with the capsulated
bacteria. The older literature contains many references to this subject,
as in the case of Wilde [485] (1896) and Beham [40] (1912); but Baerthlein [30]
in 1918 was the first to correlate the older data and to bring them into
relation with the more or less uniform colonial and cultural transfor-
mation occurring with great constancy in many bacterial species. He
showed that, while the capsulated form of the Friedländer pneumobacillus
was highly virulent, the noncapsulated form was entirely lacking in this
quality; and these types we have already observed represent the S and R,
respectively, as I also ascertained in 1925. Julianelle [505, 506] has most
recently pointed out the virulence of the S form and the nonvirulence
of the recognizable R form (noncapsulated) of the pneumobacillus. He
observed that the S type culture possessed the specific soluble substances
which were lacking in the type R culture. The situation thus resembles
that found in the pneumococcus by Griffith in 1923, and by others since
that time. Although Eisenberg [153] in 1914 had demonstrated the more
virulent character of the capsulated S form of Micrococcus (Sarcina)
tetragenus, Wreschner [492] in 1921 again studied the matter in this organ-
ism and, though not relating it in any way to microbic dissociation, gave
unmistakable evidence of the existence of the S and R types, the
former capsulated, the latter not; and also of the correlation of
virulence with the former type. This organism, it may be noted in
passing, is the only pathogenic sarcina on which we have clear data
on the dissociative forms. Wreschner demonstrated that while 0.002 oese
of the S culture killed mice in 24 hours and 0.000,01 oese killed in
four to six days, the R (noncapsulated) form was entirely lacking

in virulence when in the "absolute" R state; one-half of a slant culture killed mice in four to five days but by toxic action rather than by reason of virulence. The noncapsulated organisms were rapidly phagocytosed in the body. When, as occurred in some cases, apparently pure R culture gave fatal results it was because of its ability to undergo transformation to the S type in the body of the animal; and in such cases the S form was isolated at necropsy. When, however, the R form was no longer reversible, as indicated by tests in vitro, all virulence was lost. This form of culture he regarded as the "absolute" variant. Wreschner held, as have most others, that the capsules were the "cause" of virulence.

Although the Pasteurella form, B. avisepticus, does not properly come within the capsulated group, this organism has often been reported to form distinct capsules; and in broth cultures usually gives a viscous precipitate. The observations of Manniger [317] on dissociation of B. avisepticus have been mentioned earlier in this section, but attention may again be called to the fact that it was only the virulent or S type of the organism which possessed capsules—the nonvirulent R type lacking them entirely. The correlation of capsules and virulence is thus not limited to the members of the socalled capsulated group.

In addition to what has been said in the present section and in section 5 one concluding point may be mentioned here regarding the association of capsule formation and virulence. It has been quite commonly recognized by bacteriologists for many years that capsule formation by bacteria seems to give them a protection against the body defenses, both humoral and cellular; and that, ipso facto, capsulated bacteria are better qualified to remain in the tissues and to create infections. And this would seem to be true in infections caused by the pneumobacillus, pneumococcus, M. tetragenus and probably the anthrax bacillus. It must be pointed out, however, that a fallacy can easily be involved in this manner of interpretation. It may not be the resistance that the capsules offer to the body defense that renders more dangerous the organism possessing them. In the light of our present knowledge regarding the interrelation of dissociation, virulence and capsule formation, it appears more probable that capsules are merely correlated with virulence; and that the factors for virulence do not lie in the capsules. In other words, in the body of the animal, the pathogenic organism is not virulent because it forms capsules; but rather, it forms capsules because it is of the S type; and it happens to be the S type that is virulent. In capsulated organisms it has not yet been demonstrated that the specific soluble substances

underlying virulence and the capsular material are identical, unless we accept the recent work of Julianelle [505, 506] as evidence of this fact. Although this view is opposed to the mass of textbook opinions on the subject it finds support in the circumstance that Bail and Flammenhaft [34] could actually produce anthrax infections in guinea-pigs by means of cultures which never attained a high degree of encapsulation in the body of the animal. They conclude the matter with the following statement: "Die Ausbildung der Kapsel ist vielmehr nur eine Erscheinungsform des Infektiousität des Milzbrandes, nicht aber ihre wesentliche Ursache. Beides geht auf eine gemeinsame, aber tiefer liegende bisher unbekannte, Eigenschaft des Bazillus züruck."

Streptococcus.—In both hemolytic and greening streptococci Cowan [111, 112] (1922) in England established the association of greater virulence with the S type. In addition she pointed out the cultural, colonial and morphological differences between the R and S forms. The S type gave bluish, translucent colonies having an even outline and showing a finely granular texture. These cultures also produced an even turbidity in broth. The organisms themselves grew in shorter chains than the R form, were smaller, and showed slight variation in size. The R type, however, formed white, opaque, coarsely granular colonies with irregular outlines. The individual R organisms were distinctly larger and united into longer chains. In broth the growth occurred in the form of a precipitate. In animal inoculation tests involving these types a distinct difference in virulence was observed, although the distinction was not so clear as de Kruif had found it to be in Bact. lepisepticum, and as Eisenberg and also Wreschner had found it to be in M. tetragenus; or as others have subsequently observed it for the pneumococcus. When large doses (0.25 cc. of broth culture) of S and R streptococci were administered intravenously the comparative death rates were not far different—65 per cent for R, and 85 per cent for S. When, however, the dosage was reduced to 0.05 cc. the rate was 71 per cent for R, and 100 per cent for S. When the dosage was further reduced to 0.04 cc. the rate fell to 35 per cent for R, and 71 per cent for S. Each of these tests involved 14 mice. There was a difference, however, in the medium used in the first and second tests: in test 1 serum-broth was employed, in test 2 serum-agar slants. We know the greater tendency to dissociate in liquid mediums, and this may explain Cowan's discrepancies. In tests 2 and 3 the same medium was employed (serum-agar) and here we observe the expected conformity of results. In any case we can observe

that the R type was apparently not destitute of virulence. When the injections were not fatal, abscess formation usually occurred, and these seemed to play a part in the production of immunity. Unfortunately in Cowan's reported results we are not informed regarding the exact proportions of S and R (determined by plating) in the inoculum used. It is possible that her R strains contained mixtures of S as is often the case. De Kruif's reports are more conclusive because he presented more adequate control data on the actual S-R composition of the inoculum.

Pneumococcus.—Regarding the dissociation of the pneumococcus and its relation to virulence, although the actual facts were not fully established until the work of Griffith [215] appeared in 1923, certain earlier observations pointed clearly in this direction. Even in 1892 Kruse and Pansini [293] showed the existence of two forms of this organism: One formed chains of spherical elements with little tendency to produce capsules and possessing slight virulence; the other appeared as a lanceolate diplococcus, heavily capsulated and highly virulent. They pointed out that, while the former type was favored by growth on artificial medium, the latter was increased by animal passage. In 1891 Roger [410] pointed out that growing virulent pneumococci in immune serum rendered them nonvirulent and produced certain modifications in the type. But Issaeff,[263] who in 1892 repeated these experiments, was led to believe that such results as Roger and others had reported were due to the protective action of the pneumococcus immune serum (which accompanied the inoculum). When he took the trouble to free from the immune serum the organisms that had been grown in it, and then injected them, he found them still virulent. He thus concluded, as did many others including Metchnikoff and Sanarelli, that immune serum possessed no power to cause the attenuation of the virulent pneumococcus. Although Issaeff stated that he employed concentrated serum, we are not informed regarding the length of time the organisms were in contact with it; but one may infer that it was only to the extent of a single passage. This was apparently sufficient, however, to bring about a change in the cultural features of the pneumococcus. The growth became self-agglutinative and the organisms grew in the form of chains of many elements. Issaeff was not able to observe the curious types of cells reported by Arkharoff, although he noted that the organisms lost their capsules as a result of the growth in immune serum, a point often since confirmed.

Neufeld [363] in 1902, however, showed more conclusively that virulent pneumococci grown in immune serum lost their virulence; and, in addition, their specific agglutinability and their power to form capsules. These properties were regained by repeated passage through animals. Kindborg [509] in 1905 pointed out again the two chief types and demonstrated that the less virulent form was the one best adapted to artificial culture mediums. In 1906 Eyre, Leatham and Washburn [163] again drew attention to the two distinct types of pneumococcus as distinguished by manner of growth and by the type of lesion produced in rabbits upon subcutaneous injection—the one giving fibrinous exudates, the other cellular. The organism possessing the lower vitality on artificial mediums was the more virulent, while the form possessing the greater vitality was the less virulent. They could, however, transform the former into the latter by cultivation on artificial mediums, and then restore the original characters by passage through animals.

In 1915 Friel [185] reported that pneumococci grown in homologous immune serum became less virulent for animals; moreover, that they became at the same time more phagocytable, even in normal rabbit serum. Laura Stryker [454] in 1916 demonstrated the same influence of immune serum in producing avirulent forms of pneumococcus which thereby lost their specific agglutinating power as also their capsule-forming ability. These characters were regained by animal passage. These avirulent cultures were produced by growth in 10% homologous, pneumococcus immune serum-broth, transfers being made every two to seven days. In virulence tests on rabbits Stryker found that they often tolerated an amount of modified culture roughly a million times greater than the amount of unmodified (normal serum-broth) culture required to produce death. Fifteen cc. of modified culture was harmless on inoculation, while 0.000,005 cc. of the unmodified culture killed in 24 hours. After 55 immune serum-broth passages the culture was ordinarily so changed that it remained nonvirulent after 27 passages in plain serum-broth. The MLD of such cultures for mice was sometimes as great as 0.5 cc. while the normal virulent strain, passed similarly through normal serum-broth (control), killed mice in 0.000,01 cc. amounts. In another instance the modified nonvirulent culture was given 61 transfers in plain serum-broth and at the end of this time was still nonvirulent for mice while the unmodified culture killed in amounts of 0.000,001 cc. In still another case the modified strain was nonvirulent after 75 passages in plain broth. Although the character of reduced virulence was maintained so long as the cultures were maintained on plain culture mediums, Stryker ascer-

tained that passage of the modified strains several times through mice resulted, sooner or later, in the recovery of virulence. This point is considered further in the section dealing with the permanence of the R types (section 12).

Yoshioka [494] in 1923 demonstrated that a similar change in the form of pneumococcus could be produced by growth on unfavorable mediums, by growing at 39 C., and by drying. The changed culture type manifested itself by colony alteration; also by modified serological reactions as described in section 9. The modified cultures were particularly characterized, however, by loss of virulence. These changes did not appear simultaneously in all the bacteria of the culture but only in those of certain colonies. Other colonies remained constant to the normal and virulent pneumococcus type. Blake and Trask [52] in 1923 showed that growth of pneumococcus in immune serum produced comparatively rapid and complete changes in certain individuals but not a gradual change in all bacteria of the culture. These changes were expressed in three colony types with which were correlated variations in virulence and agglutinability, again confirming Baerthlein's fundamental postulate.

By 1922, Cowan's clearcut results on the dissociation of the streptococci, and the definite recognition of the two chief types, R and S, in many bacterial species by other workers, served to direct attention to the pneumococci from a different point of view; and in 1923 Griffith [215] in England first succeeded in demonstrating the relation of the two chief pneumococcus types with the R and S forms as earlier reported by Arkwright, de Kruif, Cowan and others for other bacterial species. Griffith regarded the S as the "unchanged culture" and R as the "variant" produced by growth in immune serum. He pointed out the colony differences (smooth and rough), and stated that, while S produced the pneumococcus "specific soluble substances," these were lacking in the R strains. While the S form, moreover, possessed high virulence (10^{-8} cc. killing mice in 2 days), the R form lacked it, even in 0.25 cc. amounts. He showed that a partial degree of attenuation existed in "partially rough" colonies; but, when these killed mice, only S cultures were obtained at autopsy. The same general results were obtained with the pneumococcus standard types, 1, 2 and 3. The subject of reversion of type is considered in a subsequent section.

In 1925 the results of Griffith were largely confirmed and extended by Reimann [405] who covered the field of pneumococcus dissociation very thoroughly; also, about the same time, by Amoss,[8] who studied the same types under the designations, C and Z. As in other cases already

reported both Reimann and Amoss obtained their dissociations primarily by growth of Pneumococcus 1 in homologous immune serum; also by growth in medium containing bile. Reimann reported the S type colonies as flat, thin, greenish, translucent, smooth, shining and sometimes "sticky;" while the R colonies were raised, opaque, less greenish, dull and sometimes dry, friable and coherent, so that they could be removed from the medium in toto, as also happens with some of the type R streptococcus colonies (Cowan). The S culture possessed the specific soluble substances and capsules, both of which were lacking in the type R cultures. The R type culture was also less easily soluble in bile. The difference in virulence between R and S was very striking. While the S type culture killed mice in doses of 10^{-6} cc., even 2.0 cc. of the R culture was without lethal effect. The virulence of "whole cultures" (S and R mixed) was less than that of "pure" S cultures. Overgrowth of the S type was induced by cultivation in normal serum or by passage through mice. The R type was stated to be irreversible (section 12). The results of virulence tests with type R as reported by Amoss agree with those of Reimann. The injection of 3 cc. killed 14 to 18 gram mice, but mice receiving 2 cc. or less survived, while 10^{5} cc. of type S culture killed in 24 hours. The serologic and immunologic characteristics of types S and R pneumococcus are presented in sections 9 and 10 of this work.

Falk, Gussin and Jacobsen [165] in 1925 showed that there existed a correlation between virulence and electrophoretic potential in variants of pneumococcus cultures. Quite recently Jacobsen and Falk [266] carried this matter further to study the potential of S and R strains obtained from various cultures through the use of immune serum added to the broth medium. The rough strains obtained as a result of 12 serial passages in immune serum-broth showed the same electrophoretic potential and virulence as the original cultures. These authors conclude that "strains of pneumococci which differ significantly in virulence are not necessarily correspondingly separable into S and R categories." From the data presented in the brief communication in question, it seems that the R characteristics were not well stabilized in the rough cultures employed. The ease with which reversion occurred might indicate that the R cultures made use of were still far from the "extreme" variant. Repetition of these studies with such stable R cultures as those produced by Reimann, Amoss or Griffith might yield different results.

Meningococcus.—Since microbic dissociation has been found to play so prominent a part in the variations in virulence of streptococci and

pneumococcus, the question of dissociation in the meningococcus naturally presents itself. That this organism is highly variable has been amply shown from the older experiments of Lepierre [510] in 1904 and others. Lepierre noted that, when fresh from the spinal fluid, the typical Weichselbaum form appeared as a gram-negative diplococcus with flattened cells; but that, after cultivation in ascites bouillon, chains appeared increasingly in successive generations, and greater growth energy developed. Accompanying this change was a loss of virulence for rabbits. The gram-reaction sometimes changed to positive. The final result was the production of a coccus resembling the Jaeger-Heubner type. Sorgente [516] in 1905 was also able to demonstrate an interchange of characters between these two types but stated that there was no change in the serological reactions. In 1909 Dopter [133] described from the nasopharynx strains of meningococcus-like organisms which were not agglutinated by antimeningococcus serum. These he called parameningococci and similar cultures were reported by him in 1911 from sporadic (but not epidemic) meningitis. Griffith [214] in 1918 reported a study of 40 strains which he investigated with reference to agglutination and biochemical reaction. On the basis of agglutination he could recognize two main groups, each of which gave its corresponding fermentative reactions. One fermented glucose more than maltose, while the other fermented maltose more than glucose. As a result of their serological studies Gordon and Murray [201] in 1915 proposed four types of meningococcus. Since that time, however, the weight of opinion seems to have been in favor of regarding a close relation existing between 1 and 3 and between 2 and 4. European workers have always laid stress on two main serologic types, following more or less the division into meningococcus and parameningococcus as proposed by Dopter. In 1920 Arkwright [15] pointed out that two main types of the organism, corresponding with Dopter's groups, had been recorded from all epidemic centers in England. Thus it appears that numerous observations concur in demonstrating that among the meningococci there exist at least two forms of culture which can be recognized by serologic and sometimes by fermentative reactions. In all these studies, however, at least up to 1915, no observations had succeeded in demonstrating the existence of any morphologic or cultural distinctions among this group; and much less a correlation of any such characteristics with such serological features as had already been pointed out. Indeed in 1918 Nicolle, Debains and Jouan,[372] as pointed out by Atkin,[21] dismissed the possibility of correlated cultural variation by the following statement:

"Considérés au point de vue de leurs charactères généraux (morphologie, aspect des cultures, propriétés biologiques), les méningocoques offrent des traits communs, que chacun connait aujourd'hui et des différences individuelles. Ces différences, légères et sans relation entre elles, ne permettent pas de créer des groupes distincts."

But already three years before this date Bernhardt [49] had made an isolated observation which, if its significance had been appreciated, would have made a fair start toward the solution of the problem of the meningococcus groups, and their actual association with cultural changes of considerable importance. Bernhardt's contribution was merely a random observation occupying but the fraction of a page in the midst of other interesting deliniations of dissociative phenomena in B. typhosus and B. diphtheriae. When three to seven day old cultures of the meningococcus were plated on ascitic-grape-sugar-agar, there arose large, flat colonies having a thinner margin in which were imbedded a wreath of daughter colonies of quite a different type. When these "Knötchen" were cultured independently, they showed a different type of growth containing large, well stained diplococci in contrast to the much smaller and poorly stained cocci in the mother culture. In the course of time the mother culture mass was destroyed, but the mutant form remained alive on the same medium up to 14 days. Eventually the mutant might revert to the mother type of culture. This observation of Bernhardt was thrown in merely to demonstrate that the peculiar type of culture reaction being described was not limited to typhosus and diphtheriae, and the study was not carried further. And so this first really significant lead to the conception of meningococcus dissociation died at its birth.

Fortunately, however, the conception of a possible correlation between serological type and cultural form of the meningococcus remained alive in the mind of the English bacteriologist, Atkin,[21] who recognized the importance of the time element in such experiments as he was about to perform. Though apparently unacquainted with the earlier work of Bernhardt, Atkin commenced a study of colony variants of various strains of Gordon's standard type strains 1, 2, 3 and 4, being at first concerned with the question of viability. Without entering into the details of this work it may be pointed out that the examination of the type 1 and 3 strains showed large, irregular, rough colonies dotted with papillae (daughter colonies), while the type 2 and 4 strains gave smaller, circular colonies having a smooth surface and no papillae. The actual colony differences are shown in Atkin's tabulation (table 1).

The details of these two cultural types, comprising respectively serologic types 1 and 3, and 2 and 4, were not further reported by Atkin in his 1923 publication; but in 1925, in his paper on the gonococcus group, he [22] presented further data. Here he calls attention to the form of growth which was characteristic of cultures coming directly from the cerebrospinal fluid of meningococcus cases. This was a thin, clear, transparent sheetlike growth, which disappeared quickly on continued artificial cultivation. On the surface of such colonies there arose, after a time, whitish papillae, sometimes discrete and sometimes clustered in masses. When these papillae were cultured separately they gave the typical whitish or yellowish and more or less opaque colony type. It was clear that the Gordon type strains which he had used for his earlier study had changed considerably to a new type of meningococcus. Here we

TABLE 1

SHOWING SOME OF THE COLONIAL CHARACTERISTICS OF ATKIN'S MENINGOCOCCUS TYPE STRAINS ON TRYPAGAR DEEP PLATES.

Type	Shape	Size	Color	Surface	Papillae	Halo*
1	Irregular	Larger	Whitish or yellowish	Rough	+	+
2	Round	Smaller	Yellowish	Smooth, glistening	0	0
3	Irregular	Larger	Whitish, pinkish	Lumpy	+ or 0	0
4	Round	Smaller	Yellow	Smooth, glistening	0	0

* A reaction product in the medium, believed to be due to precipitation of salts of legumin.

undoubtedly have the S → R (or S → O) transformation, other important aspects of which were not further reported by the author. We shall see later, however, that even the data in hand present several points of independent interest in connection with the respective colony types.

Although I have intimated above that the dissociated form of the meningococcus as reported by Bernhardt and by Atkin suggests the S → R transformation, I believe it is necessary to qualify this tentative conclusion. The assumedly R type as described by Atkin differs considerably from the sort of culture that we should expect if we reason from analogy with other bacterial species, and particularly if we have in mind the R type colonies of streptococcus and pneumococcus. Although Atkin's R form certainly marks a wide departure from the normal S type, as indicated by both appearance and serological tests, there is still the possibility that the "extreme" R has not yet been found

and that the variant represented is merely one of the intermediates in
the transition process.   One of the difficulties in its recognition lies in
the circumstance that, on the strength of any data as yet available, we
do not yet know the appearance of the transitional forms of the pneu-
mococcus or streptococcus, since these have not yet been reported.   The
clearing up of this difficulty will come only when we possess the results
of experiments in which the S type meningococcus is grown in succes-
sive passages through its homologous immune serum, since experience
with other pathogenic forms has indicated that it is by this method that
the extreme R variant can most quickly be obtained.

Gonococcus.—Regarding our knowledge of the constitution of the
gonococcus group it is becoming increasingly clear that attempts to
classify the various components in terms of standard serologic types
have met only with failure.   To be convinced of this one needs only to
examine critically the many results published during the past fifteen
years.   Neither agglutination nor absorption nor complement fixation
methods have revealed anything in the way of permanent types, and
the results obtained by no two observers have been in agreement.   In the
older body of literature, involving so much wellmeaning but hopeless
endeavor, there are, however, two investigations which have contributed
something definite to our knowledge of the group, although it is only
certain most recent studies that enable us to grasp their full significance.
The first of the two is the work of Louise Pearce [384] in 1915; the second
that of Torrey and Buckell [466] in 1922.

Pearce showed, briefly, that when a number of strains of the gonococ-
cus coming from infantile cases (vulvovaginitis) and adult cases
(urethritis) were examined by agglutination and complement fixation
methods, they divided themselves clearly into two groups correlated
with source.   Torrey and Buckell demonstrated that when several strains
of the gonococcus had been maintained on artificial culture medium for
fourteen years and were then tested by agglutination and absorption
methods, there appeared to have occurred a convergence toward a
common serological type.   They stated, "We believe that the whole
tendency under conditions of artificial culture is for reversion to our
regular group, and that a strain having attained that disposition of its
antigenic components remains in a comparatively stable condition."
These results of Torrey and Buckell, as also those of Pearce, become
further significant in the light of the most recent and highly important
study by Atkin (1925).

As already pointed out, Atkin [21] had previously shown the possibility of papilla formation (secondary colony formation) in various strains of the meningococcus, and these were subsequently observed [22] also in gonococcus. Fresh strains from various points of infection and also old laboratory strains (Gordon's original types) were grown on deep layer plates of "trypsin-broth-pea-agar" having a reaction of P_H 7.8. Under these conditions the colonies grew to a large size and after about three weeks two colony types were observed, I and II, which the reader must not confuse with the so-called standard gonococcus types 1, 2, etc. Indeed, to avoid such possible confusion, I shall refer to them as A and B, although this was not done by Atkin. Type A colonies were most prominent in material from urethritis and were large, spreading and transparent, with irregular borders. In this growth papillae finally appeared, due to secondary colony formation, sometimes few and again so numerous as to crowd the colony. In this case they coalesced into masses. The second or "extreme" colony type (which of course represented the pure growth of the papilla culture) was usually smaller, round, raised, opaque and whitish or slightly yellow; the surface was smooth (although Atkin's photographs show radial markings) and such colonies showed no papillae. This colony type was more abundant in material from old stock strains, which were sometimes composed exclusively of this form of growth. Between these two chief types, however, there were many intermediate forms which Atkin regarded as mixtures of the chief elements. Moreover there were also observed colonies showing opaque centers, but also transparent edges, in which papillae might subsequently develop.

Regarding the conditions favoring this diversity of culture form Atkin observed that alkaline (P_H 7.8) agar was necessary and that in such a medium papillae might arise in five to eight days. If the reaction of the medium was P_H 7.4 or 7.5 standard type 1 meningococcus cultures died out in three to five days leaving no papillae. When the papilla masses were subcultured it is noteworthy that the first culture obtained was not far different from the original. With further consecutive culturing from papillae, however, the papillated type of culture became increasingly stabilized and then resembled the B form. In serological tests Atkin observed that aging cultures showed a tendency to lose their agglutinability in type A immune serum; while at the same time they showed no tendency to increase in agglutinability in type B immune serum.

Atkin, whose work was purely objective, did not carry his study sufficiently far to give answers to many interesting questions; nor did he attempt to establish analogies between his results and the earlier results of Cowan on streptococcus or Griffith on pneumococcus. We can easily see, however, that the same factors for dissociation were at work in Atkin's cultures, and that he had isolated what we have come to regard as the S and perhaps R culture types, together with certain intermediate forms. He naturally concluded that his type A (I) represented the active clinical type, which Pearce had shown was the chief agent in the vulvovaginitis of children, while his type B (II) was a form more commonly isolated from adult urethritis cases, presumably in a more chronic state of infection. The convergence phenomenon observed by Torrey and Buckell manifestly finds its explanation in the circumstance that artificial cultivation, together with aging, had reduced all their gonococcus cultures to the R form in which, as we can see from numerous investigations, the antigenic similarities are always intensified. These results therefore increase the already rich evidence that the antigenic matrix which underlies type specificity is mainly, and perhaps exclusively, the property of the S form of culture.

Bacillus Proteus.—In the X19 strains of Weil and Felix we have already noted the occurrence of the two chief types of culture which Weil and Felix termed the H and O forms; and we shall see in their behavior, serologically at least, some of the characteristic features of dissociation. In addition, the existence of many "Zwischenformen" or intermediates has been noted by Felix.[172] The circumstance that it is the O type that gradually appears in old cultures, and in cultures grown at high temperatures, while it is the H type that resembles the common, spreading form, that easily gives rise to the O and carries the "double antigen," might well suggest that the H is equivalent to the S, while the O is equivalent to the R. This view was at first considered by Arkwright and Goyle,[18] but discarded in favor of the view that the H was equivalent to R and the O to S. Even this conception, however, was opposed by White,[487] who was able to present a clearer view of the situation. We shall, on a subsequent page, consider the respective merits of these presentations (section 9), and content ourselves for the moment by pointing out that it is the O form which Weil and Felix and others have shown to be the more pathogenic for rabbits. Added evidence for this might appear in the circumstance that it is the O agglutinins that appear chiefly in the serum of typhus fever patients.

Other Bacterial Species.—That there may be definite stages in the life history of still other bacterial species associated with virulence has been indicated by various writers; but the correlation of this characteristic with other features—cultural, biochemical and serological—has not made the matter sufficiently clear for us to recognize the types concerned. In the case of the cholera vibrio the relation of dissociation to virulence was perhaps indicated in the work of Lumbroso and Gerini [312] in 1911, and by Shousha [515] in 1924. Enderlein [160] also made the statement that the virulence of this species is fixed in what he termed the "anaphitit" stage.* In addition Enderlein pointed out a stage in cyclogeny marked by greater virulence in the case of M. aureus, B. influenzae, the meningococcus, the gonococcus, B. pestis, Treponema pallidum, Treponema dentium, B. tuberculosis and L. buccalis, which Enderlein places among his pathogenic species. Other instances mentioned by Enderlein have been referred to in this section—namely, the pneumococcus, B. typhosus, B. coli, Bact. pneumoniae, B. anthracis and B. diphtheriae. These statements, however, are not supported by direct evidence. The probable relation of the culture changes involved in microbic dissociation to the cyclogenic aspects of bacterial growth, founded by Enderlein on a purely morphological basis, is considered in greater detail in a later section dealing with the biological significance of microbic dissociation.†

Conclusion.—In reviewing the data presented in the present section on the relation of microbic dissociation to virulence, we perceive that many observations uphold the view that among pathogenic organisms at large virulence is not distributed evenly through all the stages of growth through which a culture passes in its normal development, but is commonly restricted to one stage of culture growth; and this, at least in many of the cases that we have examined, is what has been termed the S form. There is also evidence that the toxigenic function is similarly restricted to the same culture state. The form of culture designated the R type, on the other hand, we have commonly found to be nonvirulent, or at least less virulent than the S, although exceptions have been noted, and there are undoubtedly others. This appears not only in many references in the older literature, but also in more recent reports in which we are better assured that the chief dissociates, R and S or R and

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* For further reference to Enderlein's nomenclature in his complex exposition of comparative bacterial cytology see page 280.

† It may be noted here that the correlation between nonvirulence and the type R culture is duplicated in the lack of virulence of the SR cultures arising from bacteriophagic action. Instances of this are presented in section 14 of this work.

O, were actually concerned in the type differences. One of these excep-
tions is B. anthracis in which, so far as present observations are con-
cerned, it is the R form that carries the greater virulence. Another is
B. proteus in which it will appear (see section dealing with serologic
reactions) that the S form is not the more virulent. In the streptococci,
moreover, some degree of virulence still attaches to Cowan's R forms,
although to a much slighter degree than to type S. In this connection,
moreover, de Kruif showed a slight residual virulence still attaching
to his type R of Bact. lepisepticum for young rabbits. In both these
and similar cases it seems probable that the experiments were not carried
on with an "extreme" R form, but with one lying fairly close to S, or
perhaps containing some S elements. It must also be pointed out
that no comprehensive study has yet been made of variations in virulence
among different type S or type R strains coming from different colonies.
Moreover, we do not know that S type cultures may not lose their
virulent character without at the same time suffering transformation
into R like cultures, or into some other possible form of culture different
from either S or R; nor that some R cultures may not gain in virulence
without manifesting obvious transformation to the S form, although the
latter seems more improbable. In short, the view which the mass
of present evidence seems to support is, that both type S and type R
are to be found in nonpathogenic, as well as in pathogenic cultures; but
that, when found in pathogenic cultures, it is the S type that often carries
the greater virulence.

Regarding the underlying reason for the frequent loss of virulence
in the R type cultures, little can be said at present. It can only be
pointed out that, as shown by the work of de Kruif on lepisepticum, of
Manniger on B. avisepticus, of Friel, Griffith, Reimann and Amoss on
the pneumococcus, the type S organisms do not undergo appreciable
phagocytosis while the type R microbes are quickly consumed. In other
words S seems to possess an antagonistic action which R lacks. It seems
probable that the substance upon which this antiphagocytic action depends
is analogous to the substances designated "antiphagines" by Tschisto-
vitch, "aggressins" by Bail and "virulins" by Rosenow, and that it
is this substance that conditions virulence. It seems certain, more-
over, that it is related to the socalled "specific soluble substances"
referred to by several recent workers. The fact that these findings
regarding the relation of culture type to virulence and to phagocytability
relate particularly to a member of the Pasteurella group and to the
pneumococcus is of special interest because it was mainly in connection

with the Pasteurellas that Bail [31] and Edmund Weil most clearly established their views on aggressins and antiagressive immunity; and, as I [223] suggested some years ago, the situation in pneumococcus-pneumonia is not far different.

These matters also may have a bearing upon the problem of the nature of serum resistance and the production of the socalled "serum fast" strains reported by numerous writers, not only for bacteria but also for protozoa, and particularly for the trypanosomes. The reported observations of growth of microbes in their homologous immune serum, and of socalled adaptations thereby resulting, have been numerous; but aside from the cases already mentioned there are available few records from which we can ascertain the extent to which "serum fastness" was correlated with distinct modifications in type, or with increase or loss of virulence. From the data in hand only certain probabilities are established, and the answer as a whole must await further study. I believe, however, there are involved questions of considerable importance, not only in relation to our theories of immunity, but also in relation to our methods of practical immunization. These aspects of the matter are discussed further in section 11.

In concluding this phase of the subject it may be remarked that the relation of dissociation to the phenomenon of virulence in its broader aspects must be left an open question. For certain bacterial species, however, and among them some of the most important, I believe that it is fully established that virulence is dependent on certain definite phases or stages of culture growth; and that fully virulent and absolutely non-virulent organisms may, and commonly do, exist side by side in the same culture. In many pathogenic bacterial species at least, it now appears that exaltation of virulence is dependent upon the increase in relative frequency of S type organisms over R; and, conversely, that attenuation of virulence involves the increase in relative frequency of R type organisms over S. Whether attenuation may occur without cultural transformation to R (or O) types is still unknown, but we have no evidence of it unless it be in B. anthracis. Similarly, we have no evidence that virulence may be regained unless one of two things happens: regeneration of S culture from S elements that remain; or, transformation from R to S in cultures from which the S has quite disappeared. The second is certainly the least common, and seems never to occur in certain cultures. I believe, however, that we should hold ourselves open to the view that it may occur in all cases, provided that the proper cultural conditions are present.

In all this consideration of the relation of bacterial type to virulence, and the designation of a culture state no doubt related to that called by Enderlein [160] the "Virostadium," I believe one other reservation should be made, and this is based to a large extent on Enderlein's data. We may well hold open the possibility that all of the cultures that we have designated as belonging to the S type, and which are commonly virulent, or at least more virulent than the R forms, may not comprise a homogeneous group so far as representing a single stage in the specific cyclode is concerned. The smooth colony form of different bacterial species, we may well believe, holds within itself possibilities for variation so far as its morphological, serological and biochemical characteristics are concerned. Caution permits us to conclude only that the smooth culture type often contains, but does not necessarily comprise, the virulent forms. And I believe that the same reservation may well be made for the rough forms with respect to nonvirulence. Indeed, as already noted there are certain instances, as in the case of B. anthracis for example, in which the most virulent type of culture seems to lie in a stage of cyclogeny quite apart from the smooth type; and the same may be true of B. proteus and of the streptococci—although in the latter we know that the S form is often sufficiently virulent. In reality we have made thus far in our studies on the relation of culture type to virulence only the superficial beginning of an analysis of bacterial types which, we can scarcely doubt, will be richly supplemented and deepened by future studies. There presumably exist many pathogenic bacterial species for which the cyclogenic stage or stages possessing the more or less exclusive powers of virulence have not yet been recognized. For some this most virulent form may be a filtrable one, for our new conceptions of bacterial cyclogeny must, as indicated on a future page, be made sufficiently broad to accommodate invisible forms of bacteria as well as visible.

In final reference to the relation between culture type and virulence, it may be observed that we now seem to possess for the first time a partial explanation for common observations regarding the influence of certain cultural procedures long recognized for their effectiveness in maintaining the elusive character of microbic virulence. And here I refer especially to the use of blood, serum, or fresh tissues in culture mediums; or to the value of animal passages at least at intervals in the life of the pathogenic species. In many cases at least these methods support, or cause to develop, that particular cyclogenic stage which happens to be correlated with virulence. To what extent this conception

of the relation of type to virulence may be carried over to the toxigenic characteristics of microorganisms, as already established for B. diphtheriae, B. enteritidis and suggested for B. dysenteriae Shiga, as well as for B. botulinus, must be left for future study to demonstrate.

The significance of the restriction of virulence to the "Virostadium" of Enderlein or, as we may now term it, the virostage, in the cyclogeny of the culture, is of fundamental importance in medicine, as Enderlein has well pointed out. Indeed, I know of no discovery in bacteriology within recent years that is likely to have a more intimate bearing on many problems in immunity, susceptibility and specific therapy. We now know that, whether an organism can become dangerous to us, depends upon its ability to enter the virostage in the course of its normal progress through the cyclode. Whether it can be made to cease from its dangerous, invasive activities depends, either upon our ability to destroy it outright (which result is highly doubtful), or to force it into another cyclostage in which it is naturally harmless. The control of the cyclostage in the patient thus becomes a matter of fundamental importance, for upon it is certain to depend the issue of the infection. In another section of this work I have pointed out, as Enderlein has also suggested, that important questions will arise as to adequate methods of dealing with the virostage in specific infections; and that much future therapeutic endeavor will be concerned, not with attempts to destroy the parasite in the body by means of germicides, but with attempts to force it into another stage of development in which it is nonvirulent—or at least less virulent.* This transformation, I believe, is fundamental in the mechanics of immunity and involves one of the primary features of microbic dissociation, as will be considered further in a later section of this work.

9. DISSOCIATION AND SEROLOGIC CHARACTERISTICS, INCLUDING "SPONTANEOUS AGGLUTINATION"

Although a number of investigators as Biggs and Park, Block,[55] Delepine,[123] Fison,[177] Steinhardt,[453] and others, during the latter part of the last century and earlier years of the present, had pointed out the

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* That the further course of such a "forced" dissociation would be the same for all pathogenic organisms is to be doubted. Enderlein has already suggested that in some instances in which the virostage is identical with the socalled "Kulminante" (the peak, or point of highest development of the cyclode), the natural course would be to force the organism into a "lower" stage, marking the beginning of the return to the basic form. If, however, the virostage were represented by a form still anticipating the attainment of the "Kulminante" (which in this case we may assume to be nonvirulent) then the natural course would be to force the organism, either beyond the virostage, or into the "lower" gonidial stage which, as a resting form, according to Enderlein possesses no virulence; and with which the protective cellular factors of the tissues would react as toward a foreign body—that is, with resulting phagocytosis. It is of interest in this connection, however, that Mellon [325] has pointed out in the case of B. fusiformis that the gonidial stage in a filtrable form is able to produce infection while the common form is unable to do so.

existence of self-flocculating cultures of B. typhosus and related organisms, it was probably Nicolle [366] who first in 1898 (and later, in 1902 with Trenl [370]) presented the problem more clearly and demonstrated some of the antigenic differences in various subcultures from the same typhoid strain.   Nicolle, moreover, showed the possibility of producing the self agglutinating type by submitting normal cultures to the action of typhoid immune serum, an experiment later confirmed by Steinhardt and many others, not only for B. typhosus but for the cholera vibrio and some other organisms as well.   In 1901 the same problem was studied by Savage [425] who concluded that "clumping can be set up by a number of feeble chemical substances," as well as by immune serums.   At this time Widal and Durham had stated that there was no difference in the bacteria concerned in these reactions, although this view was opposed by Loraine Smith and by van der Velde.   Somewhat later Savage reached the conclusion that there might be some difference in the bacteria but that it was slight and ephemeral.   The correlation between spontaneous agglutination in broth and other cultural features was not made, however, until two years later when a group of observations appeared correlating the agglutinative growth with peculiar colony forms.   Among the first in this respect was Theobald Smith and Reagh [444] who in 1903 called attention to the "granular" colonies of B. icteroides on gelatin and to their agglutinating growth in broth culture.   The colony variations in B. icteroides observed by Sanarelli,[423] and pictured by him in some detail in connection with his study of yellow fever in 1897, undoubtedly concerned the dissociation phenomenon.

From this time on, many observations have been made, bearing from one angle or another, on the serological aspects of this reaction.   Since it is impossible within the brief scope of the present work to give due consideration to them all, I shall mention only those which seem to show most clearly the points at issue.   The papers referred to divide themselves naturally into two main groups.   First, those which were published before the phenomenon of microbic dissociation was clearly recognized and second, those which have appeared since that date, and which deal consciously with the chief dissociative types.   This dividing date may be placed roughly in the year 1921 when the important contributions of Arkwright [16] and of de Kruif [119] materially assisted in focussing the attention of bacteriologists upon this important aspect of bacteriological study.   It will be observed, however, that many contributions belonging in the first group have a publication date beyond 1921 by reason of the circumstance that the authors either were not aware of the publications

of de Kruif and Arkwright, or failed to grasp the significance of the results reported by them. Although some may hesitate to accept the significance for dissociation of certain examples of the earlier work, I believe there are no instances mentioned in the following review which, though sometimes slightly obscure in themselves, are not validated by later results obtained under conditions involving full recognition of the chief types of dissociates. Some others will question the exactness of the results of early work because most of it was not performed with cultures based upon single cell isolations. I am of the opinion, however, that there is little need for apprehension on this point. While it is quite true that certain aspects of study of the dissociation phenomenon require utmost accuracy so far as starting with pure lines is concerned, in the more general aspects I fail to see any essential difference in results depending on whether single cell isolation or carefully repeated colony isolations were employed; and I might add that quite typical results have been secured when, so far as we are aware, only mass culture methods have been employed.

Intestinal Bacteria.—Most of the early work involving the relation of dissociation to serologic reactions concerns the members of the colon-typhoid-dysentery and the paratyphoid-enteritidis groups, together with some observations on the cholera vibrio. Although P. Th. Müller [355] in 1903 and several others had studied the phenomenon of spontaneous agglutination of B. typhosus in immune serum, Steinhardt [453] in 1904 was one of the first to point out the relation of such cultures to colony form and to describe with great accuracy the appearance of the R type. This form of culture not only occurred spontaneously in cultures but as she demonstrated by many tests could be produced artificially through the employment of typhoid immune serum. The resulting R type was inagglutinable. Somewhat similar results were obtained on B. typhosus by Walker [477] in 1904, by Loeffler [307] in 1906 (for B. coli) and by Porges and Prantschauff [392] in the same year. Moon [351] in 1911 obtained from a single cell strain two pure line cultures one of which was agglutinable, the other not. Both bred true for many generations but eventually became similar again. Gay and Claypole [187] in 1913 obtained from rabbits in which a carrier state had been produced, a culture which failed to agglutinate in a serum that agglutinated the stock culture at 20,000. The nonagglutinating culture was, however, agglutinated by serum from a rabbit immune to the same culture grown on rabbit blood agar. Bull and Pritchett [82] in 1916 were unable to confirm these results.

In 1911 Almquist [3] observed a typhoid culture which underwent certain morphological transformations with respect to the cellular elements. Out of long rods and filaments developed small coccus forms which he regarded as gonidia. These in turn transformed into minute granular bodies (Körnchen) which were filtrable through a Berkefeld candle. These bodies refused to grow on ordinary mediums at 37 C., but grew at room temperature and at 10 C., taking the form of a yellowish layer on agar. They were not pathogenic for rabbits or guinea-pigs. When used for the immunization of rabbits, the resulting serum agglutinated not only the granular bodies but also the original culture. Such serums also possessed immunizing properties against the original culture. Almquist called the culture Bact. antityphosum. According to Enderlein's [160] terminology, one would interpret this phenomenon as the transformation of normal rod forms and filaments, first into gonidia, then into gonites. Either the smaller gonidia (microgonidia) or the gonites might have been filtrable, according to much later work of Enderlein and others.

Eisenberg [153] in 1914 obtained, among other colony mutants of B. typhosus, his socalled "Zwergform" which gave an agglutinative growth and failed to agglutinate in typhoid immune serum. Teague and MacWilliams [458] in 1917 isolated from the blood of a rabbit that had been inoculated with typhoid culture two kinds of colonies, one small, smooth and opaque, the other large, irregular and transparent. The latter gave agglutinative growth.

The results obtained in 1921 on typhoid by Morishima [352] are of special interest, and related to similar results reported earlier by Park and Williams [382] for paradysentery. Morishima showed that typhoid organisms grown on normal serum did not become inagglutinable. When cultivated on specific immune serum (rabbit) they at first became inagglutinable but later (sometimes within a few days, but always by seventy-two days) they again became agglutinable. Here we observe that the reversion seems to have occurred "in spite of the continuous subjection to the unusual environment." He used 20% immune serum in extract broth and the same dilution of normal serum for controls. In a 1:10,000 dilution the immune serum no longer produced changes in agglutinability. The author pointed out that inagglutinability was accompanied by inability to absorb agglutinin. The inagglutinable strains, moreover, showed a modified reaction to varying acidity ("acid agglutination"). No other data than the circumstance of impaired agglutinability were presented to indicated a transformation from

the S to the R type; but this transformation has occurred clearly in other cases involving a similar loss of agglutinating power under similar conditions, and thus leads us to suspect that Morishima's results concerned the dissociation phenomenon. In this connection, however, the reader's attention is directed to a curious feature of Morishima's results considered in section 12 of this paper.

Ishii [262] in 1922 in a study of spontaneous agglutination recognized the two chief types of B. typhosus and some other intestinal forms. One gave spontaneous agglutination while the other did not. Ishii did not, however, appreciate the significance of his work and failed to relate it to the dissociative process. The antigenic and serologic interrelation of the two types was not considered.

Regarding the serological aspects of dissociation among the paratyphoid-enteritidis forms fewer observations have been made. Although there may have been earlier reports in this field, Sobernheim and Seligmann [446] were among the first (1910) to demonstrate a change occurring in B. paratyphosus and involving antigenic, agglutinative and absorptive phenomena. In Baerthlein's [30] extensive report of 1918, he mentioned obtaining inagglutinable variants from all of the 14 species with which he worked. In particular he called attention to several agglutinative and absorptive differences in B. paratyphosus B and in B. dysenteriae Y. In the former case, of six colony variants, two varied in their flocculation reaction and failed to absorb the agglutinins of the other variants from specific sera. One of these two formed large, ragged colonies and was not agglutinable. The other variant was sometimes nonagglutinable and in addition possessed the ability to agglutinate spontaneously. Both of these we may now probably regard as R types. The former, Baerthlein states, after five and one-half months, gave a further variation which resembled B. typhosus in its reactions and agglutinated in high dilutions of typhoid serum. In 1918 van Loghem [308] also reported dissociation-like changes that occurred in a culture of B. paratyphosus after it had grown normally for many years. First it was observed to form indol. Later some of its variants agglutinated only slightly with the paratyphoid B serum but strongly with typhoid serum. An apparently similar transformation, but involving B. paratyphosus A, occurring under the influence of the lytic principle, was reported by Bachmann and de la Barrera.[26] In this instance a "mutant" was formed which came to manifest stronger serologic affiliation with B. typhosus than with the original paratyphoid A culture. D'Herelle,[248]

in commenting on this case, gives us to understand that the results were caused by the bacteriophage and implies that they can be produced in no other way. Such a view, however, is erroneous as we shall see in section 14 when we consider in greater detail the dissociation-provoking power of the bacteriophage. The relation of the "mutations" described by van Loghem, by Bachmann and de la Barrera and by Baerthlein are apparently all on the same order and furnish support for the conception of bacterial convergence of the R types as first introduced by Schütze and dealt with more conclusively by Goyle,[206] as will be shown on a later page.

In January, 1924, appeared an abstract account of studies by Krumwiede, Cooper and Provost [289] dealing with the serological duality of paratyphoid cultures. The authors state that they were able to obtain two components from nearly every culture examined, and that these bred true. They differed from each other agglutinatively and in agglutinin absorption and are referred to as "group components" and "specific components." The group components among themselves showed marked cross agglutination and tendency to reciprocal absorption while the specific components showed little cross agglutination and only a slight tendency to reciprocal absorption. The antiserum of either component agglutinated the other antigen only to a slight degree. The authors did not, however, in their brief report relate the phenomena observed to the dissociative reaction, although they pointed out its significance in practical serologic identification of bacterial species. They did not correlate the two components with the usual colony types although they were doubtlessly observed. These results will be considered further with reference to a later publication by Krumwiede and his collaborators.[288]

Coming now to a consideration of the dysentery group, although earlier observations had been made pointing to the dissociation of these forms, Park and Williams [382] in 1917 reported one of the clearest of the early records dealing with certain serologic changes involving the dissociative reaction. A culture of the Flexner paradysentery bacillus was transferred daily for eleven consecutive days through broth containing homologous immune serum in amounts of 15, 4, and 1.5%. The agglutinative titer of this serum preceding passage was 800. After the eleven passages in the 15% serum broth the culture showed entire loss of agglutinating power, and was reported to have lost also the ability of absorbing the specific agglutinins. The cultures grown in 4, and 1.5% serum agglutinated at 60 and 100, respectively, and continued to absorb agglutinin. The most interesting aspect of this work, however,

was the total or partial recovery of agglutinating power when the three cultures were returned to nonserum medium. The culture grown in 15% serum became agglutinable at only 200 after sixteen weeks of further cultivation while 4% serum culture returned to a 500 titer, and the 1.5% serum culture to 800 (original titer), in the same length of time. From a comparison of these results with those of Steinhardt it becomes clear that the changes observed by Park and Williams involved the S and R (or O) types of the dysentery bacillus. The reversion of the R (or O) type to the S is in accord with results reported several years later by Morishima. These have already been considered. Baerthlein [30] also has presented results on dysentery Y bearing out the work of Park and Williams, reported one year earlier.

Early observations dealing with the serologic aspects of dissociation have also been made on the cholera vibrio. Most of these deal with the modification in type determined by growth in homologous immune serum. As early as 1898 Ransom and Kitashima [501] observed that, when cultivated in immune serum, the vibrio lost its agglutinability and presented modified cultural characteristics. Similar observations were made by Hamburger [231] in 1903. The modified cultural features were such as Eisenberg [150] in 1912 clearly showed to be characteristic of what we now term the R type. The more detailed work of Balteanu [38] will be considered on a later page.

B. Pertussis.—One of the most important of the early group of studies was that of Bordet and Sleeswyk [64] in 1910 on dissociation in B. pertussis. More clearly than any other, this work demonstrated the form and the significance of the serologic changes that may accompany the dissociation reaction. Naturally, however, Bordet and Sleeswyk did not relate the phenomena observed to what we now call dissociation, or to the problem of spontaneously agglutinating cultures, which were just receiving their first serious study. This pioneer work, nevertheless, warrants presentation in some detail.

Bordet and Sleeswyk ascertained that a normal culture of B. pertussis growing on a blood medium could slowly be adapted to plain agar; but that in so doing the nature of the culture was distinctly changed. The blood culture was designated MS, the agar culture MG and their antigenic differences particularly were studied. We may omit the theoretical considerations which led to this work and turn to the results.

Rabbits were immunized against both MS and MG. The MS antiserum gave the following results: in intact form it agglutinated MS well

and MG moderately; when absorbed with MS it agglutinated neither
antigen; when absorbed with MG it failed to agglutinate MG but did
agglutinate MS. The MG antiserum, intact, agglutinated MG but not
MS. When absorbed with MS it still agglutinated MG; but when
absorbed with MG, it failed. Thus the MS antiserum influenced both
antigens while the MG antiserum influenced only its own. The serums
did not behave symmetrically. It appeared that MS antiserum contained
two agglutinins; moreover that, although MS culture absorbed two
agglutinins, it was agglutinated by only one. The authors pointed
out that the transformation MS → MG was a reversible one and very
prompt. They concluded that these differences in pertussis were com-
parable with the "natural varieties" of B. dysenteriae. They shared the
opinion of Grassberger and Schattenfroh [208] that, at least in aggluti-
nation, the immune serums do not exert their action on fundamental
substances in the bacterial protoplasm, but on "accessory substances."
They believed that such studies possessed important significance in
that it would be impossible to effect a serologic diagnosis of pertussis
if one made use of an antiserum obtained from injecting agar slant
cultures and an antigen culture grown on blood medium. The MS
culture of Bordet and Sleeswyk was unquestionably our present S type
while their MG was either the R or O in course of transformation. Their
cultures were probably to some extent mixtures of these forms, but with
such a degree of prominence of one or the other that the serologic results
were clearcut; more so, indeed, than we might expect under the circum-
stances. As we advance to a consideration of the later group of studies
in which the serologic discrepancies are found attributable to antigenic
differences definitely recognized as related to the S and R components,
we shall see that the agglutinative and absorptive scheme first clearly
pictured by Bordet and Sleeswyk may be regarded as forming the
serologic type reaction or pattern for many of the agglutination results
thus far described as involving the "double and single antigen" hypo-
thesis. The underlying facts were anticipated at an earlier date by
the studies of Theobald Smith and Reagh [445] on flagellar versus somatic
antigens (to be considered later in detail), and were further elaborated at
a later date in the work of Weil and Felix [483] on the H and O antigens
of B. proteus X19.

In view of the importance of the work of Bordet and Sleeswyk I have
reconstructed from their rather elaborate presentation a tabulation
indicating the general trend of the serologic reactions involved. From

these results slight departures may be expected, depending upon the purity of the types, the presence of intermediates, and the degree of "roughness" manifested by the R type in question; but, in general, later studies have shown a remarkable conformity to this scheme of agglutinative and absorptive reaction.

TABLE 2

SHOWING THE ESSENTIAL AGGLUTINATIVE AND ABSORPTIVE RESULTS OF THE SEROLOGIC STUDIES OF BORDET AND SLEESWYK ON THEIR TWO TYPES OF BACILLUS PERTUSSIS

(Reconstructed from their distributed data)

Antigen Culture Type	MS Antiserum Before Absorption	MG Antiserum Before Absorption
MS	Well*	0
MG	Moderate	Well
	Antiserums absorbed with MS	
MS	0	0
MG	0	Well
	Antiserums absorbed with MG	
MS	Moderate to well	0
MG	0	0

* The relative descriptive terms were used by Bordet and Sleeswyk.

In the above tabulation we may, as pointed out earlier, accept the MS antigen of Bordet and Sleeswyk as analogous to the S type ("double antigen"), while MG is analogous to the R or O type ("single antigen"). From the results presented in the table it will be clear why Bordet and Sleeswyk stated that the serological relations between MS and MG were not "symmetrical" but gave what they termed a "one-sided action."

The last paper in the older group of studies that I wish to consider is the contribution on serologic types of B. pertussis reported by Krumwiede, Mishulow and Oldenbusch[290] in 1923. On the basis of examinations of both old laboratory strains and freshly isolated strains by agglutinative and absorptive methods, they reported two distinct serologic types, A and B. Of these, most old laboratory cultures yielded both, while fresh isolations commonly gave only one. If I understand correctly the facts presented, and particularly the data incorporated in table 1 (p. 24), these two types were characterized serologically as follows: the A serum agglutinated the A antigen and also, to some extent, the B antigen. The B serum agglutinated the B antigen but had little or no effect on A antigen. When A serum was absorbed with A antigen it lost A agglutinins; also B agglutinins (?). When A serum was absorbed with B antigen the B agglutinins were lost and the A agglutinins much reduced. When B serum was absorbed with A antigen the A agglutinins were lost and the B agglutinins practically

unchanged.   When the B serum was absorbed with B antigen it lost
B agglutinins; also A agglutinins (?).   These results may be sum-
marized as follows:

TABLE   3

Showing the Essential Agglutinative and Absorptive Results of the Serologic
Study of Krumwiede, Mishulow and Oldenbusch on Their Two Serologic
Types of Bacillus Pertussis

(Reconstructed from their tabular and distributed data.)

Antigen Culture Type	A Antiserum Before Absorption	B Antiserum Before Absorption
A	2,000	0 or slight
B	1,000	2,000
Antiserums absorbed with A		
A	0	0
B	0 (?)	1,000
Antiserums absorbed with B		
A	200	0 (?)
B	0	0

Although the above authors mention the older work of Bordet and
Sleeswyk on B. pertussis, they state, "The results we have obtained
do not in any way confirm the findings of Bordet." This conclusion,
however, must be regarded as a misjudgment, probably based upon an
imperfect understanding of what the results of Bordet and Sleeswyk
actually were. If we compare the results as presented in tables 2 and 3
the similarity in both agglutinative and absorptive reactions is quite
apparent. We may therefore conclude that, although denied by
Krumwiede and his coworkers themselves, as also by Krumwiede,
Cooper and Provost[288] in 1925, the study presented by them in reality
confirms in a striking manner both the results and interpretation of
Bordet and Sleeswyk[64] relating to the existence of two antigenic
forms of B. pertussis, as first pointed out by them in 1910.

In connection with the older group of studies bearing on the relation
of dissociation to serologic reactions one other should logically be
introduced at this point; and this concerns the work of Weil and Felix
and their followers on the X19 form of B. proteus. For reasons which
will become apparent, however, it is desirable to postpone consideration
of this subject until we have examined some of the contributions mark-
ing the first of the new series of studies in which the chief cultural
types, S and R, were definitely recognized. The earliest records deal-
ing with serologic reactions concerned with clearly recognized S and R
forms as we know them today were those of Arkwright and of de Kruif,
both reported in 1921, the former dealing with dissociative reactions in
various members of the colon-typhoid-dysentery group, the latter with
the dissociation of the animal pathogen, Bact. lepisepticum. For further

reference to these studies, dealing with other aspects than the serological, other sections of this work must be consulted.

Bact. Lepisepticum.—The two cultural forms described by de Kruif [118, 119] were termed by him D (virulent) and G (nonvirulent). For our present purpose we may transpose these terms of reference to the S and R as more commonly employed. In de Kruif's experience rabbit antiserum for type S agglutinated both S and R at 2,000 after 16 hours at 55 C. The antiserum for R was less active, agglutinating S at 200 and R at 1,000. In some cases S did not agglutinate higher than 50. As de Kruif states, "the antigenic power, so far as production of agglutinin is concerned, appears to be decidedly stronger in the case of type D than of type G." Furthermore, absorption tests seemed to uphold the unity of the antigenic nature of S and R. Serum immune to the S form agglutinated S and R antigens at a titer of 1,500. But, after two hours of contact at 55 C. with the S antigen, the titer of this serum for type R had fallen to 200, and for type S to 80. The same serum, after absorption with type R, showed a reduction in titer to 40 for both S and R. De Kruif believed that R culture was not only more flocculable but also had greater binding power than S culture. He concluded that there was no "qualitative" difference in the antigenic nature of the S and R forms of culture. In the light of later work, this view demands reexamination. So far as de Kruif's results are concerned, they accord with the usual scheme at least insofar as they reveal a diversity of antigenic structure in S and R.

B. Avisepticus.—To the observations of de Kruif it may be added that Manniger [317] in 1919 had also reported evidence indicating the dissociation of B. avisepticus (Pasteurella avium), another Pasteurella closely related to, if not identical with, de Kruif's Bact. lepisepticum. He showed the production of a nonvirulent mutant from a highly virulent culture of normal type, as already indicated in section 8 of the present work. Among other points of interest he studied briefly the reciprocal serologic reactions of the virulent and nonvirulent cultures, respectively. The chief agglutinative results may be presented in the following tabulation.

Antiserums for	Antigen	Titer
Virulent	Virulent	320
Nonvirulent	Virulent	1280
Virulent	Nonvirulent	160
Nonvirulent	Nonvirulent	2560

From these results it is apparent that the agglutinability of the non-
virulent form (R) in its homologous serum was greater than that of the
virulent type (S) in either immune serum. Attention has often been
called to this peculiar phenomenon in other bacterial species, namely;
a hyperagglutinability of the R form. In my own experience, and
in the experience of certain others, the agglutination titer shown by
highly virulent fowl cholera cultures in their homologous serum is
usually low, seldom above 400 to 600.

B. Typhosus and B. Dysenteriae.—Arkwright,[16] whose excellent
report also appeared in 1921, examined nine cultures of Shiga, three
of Flexner Y, four of B. typhosus, two of paratyphosus B and three
of B. enteritidis and found that, among the Shiga cultures, all but one
gave both smooth and rough colonies which afforded pure cultures of
the S and R types. Among other cultures, some gave both S and R,
some R only and some S only. Arkwright also pointed out that various
R cultures may differ among themselves as well as from S. Although
most of the R type agglutinated spontaneously in 0.85% salt solution, a
concentration of 0.42 or 0.21% permitted stable suspensions. Shibley[440]
has employed the same method for keeping in suspension R type cultures
of streptococcus.

Arkwright inoculated rabbits with both culture types of typhoid
and dysentery and studied the reciprocal agglutinative reactions. The
S antiserums agglutinated S cultures but had little effect on R cultures.
Similarly, the R antiserum agglutinated R antigens but had little effect
on S. When a stock antidysentery serum was absorbed with S antigen
the R agglutinins were left unchanged; but when absorbed with
R antigen the R agglutinins were removed while the S agglutinins were
left intact. In other tests with B. typhosus and Flexner Y homologous
immune serums were employed. In order that these results may be
compared with others, and particularly with the results of Bordet and
Sleeswyk for pertussis, I have brought into tabular form the data
relating to Arkwright's absorption tests on dysentery Flexner Y and
its S and R forms.

From the results of the serologic tests presented above dealing with
the R and S forms of dysentery Flexner, it can be observed that,
although insufficient absorptions are manifested, the general picture
resembles somewhat that given by Bordet and Sleeswyk, and by
Krumwiede for pertussis; also as we shall see, by Griffith for the
pneumococcus and by Smith and Reagh for the motile and nonmotile

forms of the hog cholera bacillus. I believe that these examples are sufficient to demonstrate the fact that, serologically, the R type culture is not a random variant or mutant as many have supposed, but a definite form or stage of culture which stands in a definite serologic relation to the·S form; moreover, that this serologic relation is not variable for different bacterial species, but highly constant, in whatever species the two dissociates may be discovered. We shall see later how this conception, complicated somewhat by the introduction of a third clearcut antigenic type, underlies the complex structure which Weil and Felix and their followers have raised about the diversified H and O forms of B. proteus X 19.

TABLE 4

SHOWING THE RESULTS OF ABSORPTION TESTS WITH SERUMS IMMUNE TO TYPES S AND R OF B. DYSENTERIAE FLEXNER Y.

(Compiled from data in Arkwright's table 7, p. 47.)

Antigen Culture	S Antiserum	R Antiserum
	Before Absorption	Before Absorption
S	20,480	160
R	320	2,560
	Antiserums absorbed with S	
S	1,280	0
R	160	1,280
	Antiserums absorbed with R	
S	10,240	0
R	160	160

Another contribution of interest referred to in greater detail in section 11 is that of Ørskov and Larsen,[513] mentioning certain serologic aspects of dissociation in a paradysentery culture. From the stock culture were isolated two chief types, first recognized by colony forms, which they termed "V" and "B." Both of these variants gave agglutinating serums while culture M (a further variant of V) gave a serum with no agglutinating power. The detailed serologic results of their study show that V antiserum agglutinated V well and M slightly; B antiserum agglutinated B and Bu (a further variant from B), but not V or M; and M antiserum agglutinated M only slightly and V, B and Bu not at all. Furthermore, absorbing the V antiserum with V antigen removed all V agglutinins, while absorbing with culture M left fair agglutinating capacity for V. Both the direct agglutination and the absorption tests are incomplete. They serve to indicate clearly the diversity of serologic type but without at the same time making clear the exact nature of the variants. What data are available, however,

suggest that V was a type S, that B was an intermediate and that M was a full R and Bu an intermediate fairly close to B. The results of fermentation tests support these conclusions. On the whole, these results of Ørskov and Larsen, although lacking in analysis on the part of the authors, add evidence for the conception of the heterogeneous nature of the R type.

Salmonella and the Aertrycke Bacillus.—Andrewes [10] in 1922 obtained two different serologic forms from a single strain of a member of the group, Salmonella. One of these produced, and reacted with, the group agglutinins, while the other showed as dominant the specific agglutinogens and reacted to the specific agglutinins. In this case, however, the two colonies from which these cultures came were reported as alike in their morphologic characters. Griffith [24] described what was probably a similar case for the meningococcus and expressed his opinion that both of these instances concerned an incomplete S to R transformation.

The studies of Schütze [436] in 1922 on dissociation phenomena in a culture of the Aertrycke bacillus followed closely the lines established by Arkwright one year earlier. Here he found a variant whose agglutination and absorption relations to the original culture were lost. Serum immune to what we may regard as the S culture would not agglutinate the R form, nor the R serum the S culture. Such extreme diversities are not common in the literature. In certain tests the R culture was not agglutinated even by the R serum itself. White [487] made a similar observation for a certain R strain of Salmonella.

But perhaps the most important aspect of Schütze's work was the observation that the R type culture of one bacterial species may be agglutinated even to titer by the R immune serum of other species, even when the S forms of the same cultures reveal no or slight serologic relation. For instance Schütze states: "rough variants of Gaertner's bacillus, paratyphosus A and typhoid strains will agglutinate, sometimes to the titer limit, with rough sera from the paratyphoid B group, while smooth prototypes, from which they have been derived, remain quite unaffected." This phenomenon the author refers to as a sort of "serological cosmopolitanism" among the rough cultures. This observation, I believe, constitutes the first clear recognition of the relation of the dissociative reaction to the phenomenon of bacterial convergence. The phenomenon has parallels elsewhere in the literature. For example it appears in Laura Stryker's [454] and Yoshioka's [494] cases of pneumococcus

dissociation (although the phenomenon was not recognized in either instance) ; also in Reimann's [405] case of the agglutination of the R type pneumococcus in heterologous pneumococcus immune serums; also presumably in Torrey and Buckell's [465] convergence of their types of gonococcus. It was also no doubt concerned in the divergent serologic reactions of the B. paratyphosus A of Bachmann and de la Barrera [26] as already reported. In all these instances, some of which remain to be considered, we can observe the phenomenon of bacterial convergence which manifestly depends on similarities in antigenic structure among the R forms; and which, according to the more recent study of Goyle,[206] may sometimes appear in relation to the intermediate (O) culture types, as will be shown in greater detail on a later page.

B. Paratyphosus and Related Organisms.—In 1925 Krumwiede, Cooper and Provost [288] presented in their comprehensive study of agglutination absorption various references to the serological behavior of certain variants, some of which were recognized by the authors themselves as R types. Still other variants, in the light of much earlier work, were manifestly intermediates, or "incomplete" R forms. Much of the work of these writers dealt with B. paratyphosus in which they could recognize three chief colony types, smooth, mucoid and rough (p. 135). The mucoids, as Fletcher [179] also had observed, were especially common in carriers and convalescents. Platings direct from feces often gave mixtures of S and mucoid, at other times mixtures of S and R. The rough forms were much more stable than the S or the mucoids, although all tended to breed true in plating as well as in passage through animals. The mucoid type might develop either S or R, a point to which we shall return in our consideration of the intermediate type. The R cultures showed greater agglutinability and grew as a sediment in broth.

Regarding the actual serologic characteristics of these types the authors present many tables giving the results of direct agglutination and absorption tests. With a few exceptions, however, the results are unusual and entirely out of harmony with nearly all other work on the serology of the S and R forms. They were able to detect no uniform difference in regard to the antigenic nature of the rough and smooth strains (p. 145) and concluded that colony variation was not a fundamental factor in agglutination results, with the possible exception of the mucoids. The state (p. 147), "In most of our work the rough and smooth varieties have been serologically similar." When their

results were occasionally in agreement with those of other workers
(Weil and Felix, Arkwright), they believed that "other variation fac-
tors" ("degradation," "vertical variation," "lateral variation") were
involved in the process. They concluded that the rough colonies
possessed a "duality" similar to that of the smooth (p. 171). They
criticized Baerthlein's work on colony variation and correlated sero-
logical features as inconclusive and "difficult to understand" (p. 200).
They doubted the reliability of the method of "aging" cultures for the
production of colony variants as employed by Baerthlein and numerous
other workers, and questioned whether this procedure "actually induced
variation or permits a survival of existant variant individuals" (p. 198).
They could not accept the results of Bordet and Sleeswyk,[64] on serologic
variability of B. pertussis, as previously mentioned, and believed the
differences observed were due to "pseudo-reactions" (p. 203). They
considered Stryker's [454] detailed and thorough study of the pneumo-
coccus variants (later fully confirmed by Griffith,[215] Yoshioka [494] and
Reimann [405]) to be too brief to warrant interpretation "from our point
of view" (p. 203). Regarding Griffith's work on pneumococcus serology
as related to dissociation, these authors found it "impossible to tell how
far some of the agglutination results were due to differences in reactivity
and how far to degradation" (p. 203). Regarding the suppression on
agglutinability determined by cultivation in homologous immune serum,
as fully demonstrated in numerous cases in recent years (see later pages
in "Incitants"), they state: "as far as we know the changes observed
were not associated with proven antigenic change" (p. 204). They did
not believe "that the rough colony variant is also a serologic variant"
(p. 209).

   A few points considered are however constructive, although they
have been developed more clearly by other workers, both before and
after the appearance of Krumwiede's study. They showed clearly that
their "degraded" variants were characterized by an antigenic loss, such
as also characterizes the O proteus type. They realized, moreover,
that "degradation" might be followed by the acquisition of a quite new
antigenic capacity not present in the original culture (p. 196). This
was also pointed out by White [487] in England in 1925 and confirmed
by Goyle [206] in 1926 with a great wealth of convincing experiment.
Krumwiede and his collaborators also noted the parallelism between
paratyphoid antigen heated, and the naturally "degraded" antigen
(p. 197). As we shall observe a little later the explanation of this
similarity (which is seen also in the relation between the H and O types

of Weil and Felix) was presented by Goyle in 1926. Krumwiede and his collaborators also note the fact that the antigenic basis for common agglutination reactions lies in what they term the "group component" (probably the pure O or R antigen), and also in the antigen of the "degraded" variant. This point was definitely proved for typhosus and enteritidis by Goyle in his comprehensive work in 1926 dealing with the serologic aspects of bacterial convergence.

With these few exceptions, the work of Krumwiede and his collaborators dealing with the serologic aspects of dissociation shows a striking lack of conformity with the mass of recent studies. Although the authors failed to comprehend either the significance or the practical aspects of dissociative reactions, as indicated by the curious nature of their criticisms of other contributions which in reality attack the problem more directly, the points covered by them clearly dealt with the phenomenon of microbic dissociation. Their "degradation variants," "lateral variants" and "vertical variants" easily find a place in the system, although this sort of terminology is both confusing and misleading.

In concluding this consideration of the work of Krumwiede and his collaborators I may add that the chief obstacle that probably prevented them from more completely recognizing the underlying phenomenon of antigenic dissociation was the circumstance that they did not work with well stabilized R types and failed to recognize the wide latitude that always seems to characterize the cultural, morphologic and serologic expression of these forms, depending on the manner of their production and the degree of their stability. In addition, Krumwiede's work serves as an excellent example of the future necessity of providing a preliminary groundwork in the simple phenomena of dissociation before attempting the difficult antigenic analyses relating to this reaction.

B. Diphtheriae and Hofmann's Bacillus.—Although several investigators have studied the serologic relation of clinical diphtheria cultures to the types of Hofmann, the results have been variable. In the main there has been observed an apparent lack of relationship. Among the latest workers in this field are Bull and McKee [81] who in 1924 studied the subject by complement-fixation methods. They observed that cultures of B. diphtheriae and Hofmann strains could be clearly distinguished by means of such tests and concluded (p. 107) that "the common parentage of all strains of B. hofmanni and B. diphtheriae cannot be established by means of the

complement-fixation reaction." This and similar instances serve to illustrate a fallacy which has been very common in dealing with the serologic relationships of bacteria. It involves the erroneous assumption that different colony strains coming from the same pure line must show a high degree of serologic homogeneity. This is well illustrated by the statement of Eberson,[145] who became involved in difficulty over this point (see Mellon[327]). Eberson states (p. 28): "Bacteria are unicellular organisms which are produced from other like organisms by the process of fission—an act in which the entire parent substance may be said to divide itself equally into two fractions, both of which constitute the resulting pair of bacteria. Under such conditions the offspring cannot possess what was not present in the parent." Thus Eberson, as also many others, is led to assume that, if two cultures, descended from the same assumed pure line, do not manifest symmetrical serologic reactions, a conclusion that the two forms may have a common parentage is scarcely justified. If there is one significant point in the serologic aspects of microbic dissociation it is the fact that there is no necessary symmetrical serologic relationship between different daughter colony strains. Indeed, when we deal with the R and S forms of bacteria, as will be shown in future pages of this section, the expectation is that the serologic reactions of the rough variant, as also certain other variants, will be quite unlike those of the parent form; and may be quite unlike the reactions of sister colony cultures taken from the same plate. The time is past when lack of serologic symmetry must necessarily be interpreted as indicating lack of "common parentage"; and this holds true whether the tests involve agglutination, precipitation or complement-fixation reactions. In other pages of this paper is presented evidence favoring the view that the Hofmann types represent an R form of the clinical diphtheria culture type. Under these circumstances a symmetrical serologic relation with the clinical form would never be expected. The criterion of relationship or nonrelationship which Bull and McKee seek to employ is therefore of no value for eliciting the information which they demand regarding the position of these two bacterial types.

Pneumococcus.—In the pneumococcus serologic disparities have been reported many times, beginning perhaps with Neufeld[363] in 1902. In most cases these have related to "two different types" of the organism, differing not only in cultural and colonial features (though these were recognized clearly only at a later date), but also in serologic char-

acters and virulence. Neufeld reported a certain pneumococcus strain, agglutinating well in immune serum, which suddenly became non-agglutinative and at the same time nonvirulent. He also observed that serum immune to a certain virulent strain agglutinated foreign virulent strains, but not a foreign strain which was from the beginning non-virulent. Strains that agglutinated well lost this ability when grown for some time on agar.

In 1916 Laura Stryker [454] carried out an investigation of funda-mental significance dealing with pneumococcus variability determined by growth of the virulent organism in homologous immune serum. Although she did not recognize the fact of dissociation, merely observ-ing that "some biologic change must have taken place" in her cultures, the data presented in her paper clearly indicate that she was concerned with the dissociative phenomenon as more clearly recognized by Griffith, Reimann and Amoss some years later. Her results merit report in some detail.

Stryker grew virulent pneumococci in 10% homologous immune serum broth in continuous series, transferring every two days or every week. Control cultures were carried in normal serum broth. The first change noticed in the normal culture involved a sedimentary form of growth in the immune serum medium. The sediment was in the form of a hard mass and was broken up only with difficulty. After five or six transplants in the same medium, however, the sedimentary growth became flocculent and was easily broken up by shaking. Type 2 pneumococcus culture required a longer time for this change to occur. In both types the form of the individual cells was changed to threads or to swollen cells, often clumped. In this modified culture state some of the characteristics of the normal culture remained: gram-positive staining, solubility in bile, and fermentation of inulin. Other char-acteristics, however, changed: capsules were lost, virulence was lost and the antigenic and opsonic reactions were modified; the culture agglutinated with greater difficulty and phagocytability was increased.

Regarding the culture growth, this lost the moist, confluent and greenish appearance of the normal pneumococcus culture and grew in the form of dry, brownish colonies which showed a tendency to pro-duce hemolysis. In broth they gave a sediment which still persisted after 25 passages in a plain medium. Regarding agglutination in immune serums, there was observed a tendency to develop nonspecific agglutinins. Serum immune to the modified culture (type 2) aggluti-nated the modified strains of both types 1 and 2, but failed to agglutinate

normal strains. Serum immune to the normal type 2 agglutinated the homologous normal strain and also, though to a slighter degree, the modified cultures of types 1 and 2.

In absorption tests, Stryker showed that when antiserum for a normal type 1 or 2 culture was absorbed with type 1 or 2 modified strains, the agglutinins for both homologous and heterologous modified strains were removed, while agglutinins for homologous normal strains remained. When, on the other hand, antiserum for a normal type 1 or 2 culture was absorbed with normal homologous strains the agglutinins for both normal and modified strains were removed. Here again we have a striking similarity to the early case of Bordet and Sleeswyk involving B. pertussis. These serologic results, Stryker assumed, were due to a suppression of certain receptors, as Cole earlier believed to be the case in certain serologic disparities with B. typhosus. We can see clearly, however, that they are in line with many other instances of the same sort dealing with the socalled "double" and "single" antigens. Other interesting points in Stryker's paper are considered further in sections 11, 12 and 13 of the present work.

As stated earlier, although the splendid work of Stryker has given one of the clearest pictures of the serologic aspects of the dissociative reaction in the pneumococcus, she did not recognize the biological significance of the phenomena observed. We shall see presently, however, that our view of dissociative changes in this case is amply justified by the results obtained somewhat later by Griffith, by Reimann and by Amoss, working with recognized R and S forms of the pneumococcus.

In 1919 Mildred Clough [102] examined many strains of the same organism and observed nine which precipitated with types 1, 2 and 3 serums. These atypical strains were slightly virulent and highly susceptible to phagocytosis. None of the nine absorbed precipitin from type 1 or 2 serums, and serums immune to these strains agglutinated these strains only. Nicolle and Debains [371] according to Eastwood [143] found among many typical pneumococcus cultures about 30% which agglutinated with several of the type serums, but mainly with type 2 serum. Yoshioka [494] in 1923 presented a very clear picture of pneumococcus serologic variability involving dissociative aspects. He showed that whenever typical and virulent pneumococci were placed under such conditions as determined a loss of virulence (as drying, unfavorable medium, incubating at 39 C., etc.), such cultures also experienced marked antigenic changes. These changes involved not only loss of agglutinating power in their homologous, immune serums

(i. e., homologous to the unmodified culture type), but newly acquired agglutinability in heterologous serums. What is perhaps more important, however, they had also gained a susceptibility to equal agglutination with serums immune to certain streptococcus forms (Aronson virulent). Assuming, as I believe we justifiably may, that these pneumococcus variants represented either R types, or cultures in the course of transformation toward the R type, we have here another interesting case of the "serological cosmopolitanism" of the R forms as developed by Schütze [436] for certain intestinal bacteria. In the light of this and other work, there can be small doubt that we shall soon find means to transform any of the standard pneumococcus types into quite different serologic forms. Indeed, Berger and Engelmann [48] have already reported such a transformation of a type 3 pneumococcus into type 2. What we mainly lack at the present time with reference to the serology of the pneumococcus is a thorough study of the relation of the various types to the distribution of the fundamental heat stable and heat labile antigens.

The several studies just reported have served to give us an excellent picture of some of the results of dissociation in the pneumococcus; but it remained for Griffith [215] (1923) in England, following soon the important lines established by Arkwright and de Kruif, to correlate many of the curious points in the behavior of this organism with the definitely recognized products of dissociation; namely, the S and R culture forms. The serologic characteristics of these variants, although according well with the findings in other bacterial species, merit presentation in some detail. Other aspects of Griffith's work are presented in other sections of this work.

To state the matter briefly, Griffith produced the R form of the pneumococcus by growing normal, virulent cultures in their homologous immune serum. These modified strains were noncapsulated and were lacking in virulence. It is their serologic characteristics, however, that are of present interest. Rabbit serum immune to the S form agglutinated S and R about equally, but only at a titer of about 160. The R antiserum agglutinated R at 640 but S scarcely at all (10). When S antiserum was absorbed with S antigen it lost agglutinins for both S and R culture. When S antiserum was absorbed with R antigen it failed to agglutinate R but still showed some agglutinating power for S. When R antiserum was absorbed with R antigen (twice) it gave no agglutination of R antigen (it always lacked agglutinative power for S). When R antiserum was absorbed with S antigen its

titer for R dropped from 640 to 160. Here we have clearly the same "one-sided action" described so well by Bordet and Sleeswyk for B. pertussis in 1910. In order that the comparison may be facilitated, I have brought together in tabular form Griffith's essential serologic results.

TABLE 5

Showing the Essential Agglutinative and Absorptive Results of Serologic Studies of Griffith on the Recognized S and R Types of the Pneumococcus

(Reconstructed from Griffith's distributed data.)

Antigen Culture Type	Type S Antiserum	Type R Antiserum
	Before Absorption	Before Absorption
S	160	0
R	160	640
	Antiserums absorbed with S	
S	0	0
R	0	160
	Antiserums absorbed with R	
S	"Moderate"*	0
R	0	0

* Griffith does not state exact titer.

Griffith also presented interesting results of precipitin tests which other reports lack. For antigens, Berkefeld filtrates of broth cultures were employed. When S antiserum was added to S filtrate there formed a copious precipitate characterized by firm masses which could not be easily broken. When the S antiserum was added to R filtrate no precipitation occurred. When the R antiserum was added to either S or R filtrate no precipitation occurred in either case. These results seem to indicate that the precipitinogen was absent in the R cultures, and that the respective serum failed to possess a rough precipitin. Thus, the precipitin seems to be related to those antigenic groups which distinguish the S type of culture and also underlie the characteristic of virulence. The discrepancies between precipitin and agglutinin reactions in general are, on the strength of such findings if confirmed, likely to afford an interesting field for further study.

Although we owe to Griffith the first clear recognition of the S and R types of the pneumococcus and the demonstration of the association of the "specific soluble substances" with the S form, as shown in the section on "Dissociation and Virulence," other interesting features of pneumococcus dissociation were added by Reimann[405] in 1925 supporting the view of "serological cosmopolitanism" of the R types already introduced into the field of pneumococcus dissociation by Stryker and by Yoshioka. Reimann ascertained that, when serums of types 1, 2 and 3 were applied to the S and R forms of a type 1 pneumococcus

strain, the S culture agglutinated in type 1 serum only, while the R culture agglutinated in all three standard type serums. The "pure" S form was thus "type-specific" while the R form was not. According to Reimann's interpretation, and as Griffith had earlier stated, the R form of culture had lost the specific soluble substances. Reimann's results thus confirmed a number of preceding observations pointing to the heterologous nature of the R type in its serologic behavior. The serologic interrelation of the S and R cultures was not considered by Reimann, nor was it mentioned in the paper by Amoss,[8] although the latter presents certain brief immunologic features of pneumococcus dissociation to be mentioned on a later page.*

* Since the writing of the foregoing pages there have appeared two valuable papers by Julianelle[505, 506] dealing with the antigenic structure of Friedländer's pneumobacillus and including interesting data on the serological relations of the S (capsulated) and the R (noncapsulated) forms of culture. Julianelle observed that it is only the S type which produces the specific soluble substance, analogous to that noted by Griffith, Heidelberger and Avery and others in the pneumococcus. This substance, lacking nitrogen and appearing to be of the nature of a polysaccharide, seemed to endow the culture with the virulence characteristic of the S type. This carbohydrate when separated from the cells was nonantigenic; but in the form in which it existed in the cells was strongly antigenic, and upon injection gave rise to an antibody specific for the type (A, B, C or X) of the pneumobacillus. In this manner it seemed to condition the "type-specificity." This carbohydrate was found to be lacking in R type cultures and in the substance of S type cultures the cells of which had been decapsulated by the acid method of Porges. The remaining antigen, as also the dominant antigen of the type R cells, appeared to be a nucleoprotein which was highly antigenic, giving rise to distinct antiprotein antibodies. The serologic reactions involving these two different antigens (carbohydrate and nucleoprotein) were as follows:

Serum immune to the S type culture contained antibodies which "cause the type-specific agglutination of encapsulated cells, precipitate the carbohydrate derived from organisms of the homologous type, and afford passive protection in white mice against infection with bacilli of the same type." S antiserums were found to contain negligible amounts of antiprotein and agglutinated only irregularly cells of the R type.

Serum immune to type R culture, or to the nucleoprotein constituting its chief antigen, agglutinated or precipitated R culture or R protein, but did not react with the type S cells or with the polysaccharide. Such R serums reacted not only with the homologous R culture, but also with decapsulated cell substance and with any heterologous type R strains; also with the nucleoprotein of B. coli, B. aerogenes and the granuloma bacillus, thus manifesting the "serological cosmopolitanism" noted elsewhere in this work. It may be added here that Julianelle's conception of the nature of the type R culture is that of a "degraded" form of the organism, and in this respect his view coincides with that of Krumwiede. Although neither entertains notions of microbic dissociation in the biological sense, the work of Julianelle indicates in an especially clear manner the extent to which thorough-going antigenic analyses may lead us closer to the chemical realities underlying the dissociative reaction.

Meningococcus and Gonococcus.—In the section dealing with dissociation and virulence, I have pointed out the interesting data secured by Atkin [21, 22] in the colony dissociations of these diplococci. Although we have little knowledge of the serologic interreactions between the clearly recognized types, either in meningococcus or in gonococcus, it is desirable to mention one other matter bearing on the often observed relationship between certain strains of these two species. Early observations dealing with this subject were introduced in 1906 by Bruckner and Cristéanu [74] and by Vannod,[471] followed by those of Wollstein [491] in 1907, Teague and Torrey [459] in 1907, Gurd [219] in 1908, Dopter and Koch [135, 136] in 1908, Arkwright,[12] also Elser and Huntoon [157] in 1909, Colombo [107] in 1911 and by Arkwright [13] in 1912. In a general way, as Arkwright has pointed out, the records of these investigators have demonstrated that a marked similarity exists between certain strains of the two species from the point of view of agglutination, complement fixation and the generation of antibodies. He stated: "The differences between the individual races of gonococcus, or of meningococcus as tested by these methods, appear to be almost as great as between the two species." And again: "It is therefore unlikely that well-marked constant differences along these lines will be found between the groups and subdivisions of the whole class to which the Gonococcus and Meningococcus both belong" (l.c., pp. 116 and 117). Wollstein [491] who worked with monovalent serums was able to observe no difference between many meningococcus and gonococcus strains. Also Colombo, who studied the relation particularly by complement-fixation methods, was unable to discover specific differences. Arkwright,[13] who considered the subject further in 1912, came to the following conclusions: "Meningococcal sera produce complement fixation as readily with some gonococcal extracts as with the extracts of some strains of meningococcus; whereas no reaction is obtained with some heterologous meningococcal extracts. A monovalent serum usually reacts better with an extract of its homologous coccus than with extracts of other strains of Meningococcus or Gonococcus, but a gonococcal extract sometimes gives a better reaction with a meningococcal serum than the homologous extract does." . . . "No satisfactory distinction between Meningococci and Gonococci can be demonstrated by means of complement fixation tests."

From the facts presented above it becomes clear that the serologic relation existing between the meningococcus and the gonococcus parallels to a degree the serologic affinities manifested by the R forms

of culture of several members of the colon-typhoid-dysentery group as also of the pneumococcus-streptococcus group; and thus seems to suggest the existence of a sort of bacterial convergence—at least with respect to the serologic reactions. Atkin has already presented data which indicate the facility with which freshly isolated meningococcus and gonococcus cultures dissociate into the intermediate or into the R type; also the probable frequency of this reaction, after they have been grown for a time on artificial culture mediums. From this it seems probable that it is through the medium of the O or R forms of culture that the somewhat noteworthy serologic affinities between the meningococcus and gonococcus are maintained. With our present knowledge of the fact of dissociation in these species, and the means by which it may be brought about, the problem is now open to experimental attack. Such a study is likely to reveal more closely than heretofore the nature and cause of the serologic "discrepancies" so frequently observed; and perhaps in this way to establish on a firmer basis our methods of serologic diagnosis of meningococcal and gonococcal infections.

B. Cholerae Suis and the Hypothesis Relating to the "Flagellar" and "Somatic" Agglutinative Antigens.—Before continuing further the serological aspects of dissociation, it is desirable to consider its possible relation to one of the theories of agglutination current in earlier years, and actively revived in recent time in connection with the "double" and "single" antigen hypothesis. As we shall see later, this problem has a bearing on the H and O forms of B. proteus first described by Weil and Felix while in this country it had been elaborated much earlier at the hands of Theobald Smith and Reagh [445] in 1903 and by Beyer and Reagh [50] in 1904. Already in 1897 Malvoz,[316] and one year later Dineur,[128] had called attention to the possible rôle played by the flagella in agglutination, a conception also furthered by Nicolle and Trenl [370] in 1902. Smith and Reagh accordingly undertook to demonstrate the separate nature of flagellar and somatic antigen, using for this purpose certain cultures of the hog cholera bacillus. Although all common strains of this organism are motile, they had at their disposal a nonmotile strain. They stated, however, that between this strain and their other cultures there was no difference except in motility. First they showed that the action of rabbit serum immune to the motile type (M) on the type M antigen was different from the action of the serum immune to the nonmotile culture (NM) on the nonmotile form. These

differences were as follows: the reaction was delayed; the clumps were in the form of small, compact granules; loose clumping, characteristic of the motile form, was absent; to obtain an active serum against the NM form required a long and intense immunizing procedure. All of these points, as the combined results of many investigations have shown, are characteristic of intermediate or R type cultures. The results of cross agglutination tests with the two immune serums are tabulated.

TABLE 6

SHOWING THE RESULT OF CROSS AGGLUTINATION TESTS BETWEEN SERUMS IMMUNE, RESPECTIVELY, TO THE MOTILE AND NONMOTILE TYPES OF SMITH AND REAGH'S HOG CHOLERA CULTURES

(Assembled from Smith and Reagh's distributed data)

Antiserums for	Antigen	Agglutination Titers
Motile form (S ?)...	Motile	10,000
	Nonmotile	500
Nonmotile form (R or O*)...............................	Motile	20-200
	Nonmotile	200-500

* See "O" under proteus dissociation, p. 144.

These results show a distinctly characteristic "one-sided action." Next, an incomplete series of absorption tests was performed with the serums absorbed with M and NM antigens as shown below.

TABLE 7

SHOWING THE RESULTS OF ABSORPTION TESTS WITH SERUMS IMMUNE, RESPECTIVELY, TO THE MOTILE AND NONMOTILE STRAINS OF SMITH AND REAGH'S HOG CHOLERA CULTURES

(Assembled from Smith and Reagh's distributed data)

Antiserums for	Antigen Absorption	Titers after Absorption	
		Motile	Nonmotile
Motile form....................	(Unabsorbed)	20,000	200
	Nonmotile	20,000	100
	Motile	"decided loss"	"decided loss"
Nonmotile form...............	(Unabsorbed)	500	500*
	Nonmotile	(No data)	(No data)
	Motile	40	40

* More nearly typical results are shown in table 6.

From these results the authors conclude that the agglutinins are differentiated into "flagellar" and "body" (somatic) types, and that therefore the motile bacteria carry a double antigen while the non-motile carry a single. This view has been reinforced by the further study of Marion Orcutt,[377] in 1924, in which she reported having separated the actual flagellar antigen in pure form. Although the experimental data on agglutination and absorption presented by Smith

and Reagh and their immediate followers are too incomplete to make possible a satisfactory comparison with other work involving the "double" and "single" antigens, it is nevertheless apparent that there exist similarities with the data presented by Bordet and Sleeswyk and others. We know, moreover, from the statement of Smith and Reagh that the agglutination of the nonmotile culture was in the form of small, compact granules; and this phenomenon, as has been shown by Goyle for B. typhosus and B. enteritidis, as described by Weil and Felix for B. proteus X19 (to be mentioned presently), and by Balteanu[38] for Vibrio cholerae, is characteristic of the intermediate or O type of culture—perhaps sometimes of the R. It therefore seems possible that the nonmotile hog cholera culture of Smith and Reagh represented such an O form, or a mixture of O with the variant, R. The motile form, on the other hand, was unquestionably the normal S culture. The true R type of the hog cholera bacillus is probably that described by Orcutt[376] at a later date.

While the view of flagellar as opposed to somatic antigens and agglutinins elaborated by Theobald Smith and Reagh and their followers on the primary postulates of Malvoz is sufficiently alluring and has been maintained even to the present time by many workers (including Felix,[172] Braun and Schaeffer[66] and others for B. proteus and by Feiler[167] for B. typhosus, as well as by Furth[188, 190] for typhoid, paratyphoid and dysentery bacteria), certain modifications have been required. Since it was found that certain nonflagellated bacteria could be split into two forms, R and S, giving exactly the same order of serologic reactions as those observed in the R and S types of motile bacteria, the flagellar hypothesis could scarcely stand. When the new results concerned capsulated bacteria such as the pneumococcus, the S type of which is capsulated while the R form is not, it was naturally logical to modify the flagellar hypothesis into a capsular hypothesis; and this was quickly done. But, if we go a step further we encounter exactly the same sort of serologic disparities in the behavior of organisms that are not noteworthy by reason of possessing capsules, namely, B. pertussis, the streptococcus, and Vib. cholerae. Among these we can also observe the same sort of "one sided action." We are therefore driven to dispense, not only with the old flagellar hypothesis, but also with the capsular hypothesis. When, as is certainly now the case, we are pushed to the morphologic extremity of our arguments, we may perhaps fall back upon the recognition of ectoplasmic versus endoplasmic substances to explain the phenomenon. It is possible that here

we may yet make a stand.   I regard it more probable, however, that we
shall eventually come to disregard all distinctly morphological elements
in respect to differences in antigenic behavior and begin to study sero-
logic differences in terms of the presence or absence of certain specific
soluble substances wherever they may have their point of origin in the
bacterial cell, and with whatever cell structures they may seem to be
related.   Griffith has already made a beginning in this direction in his
observations relating to the specific soluble substance of the pneumococ-
cus in cultures of the S type.*

Micrococcus (Brucella) Melitensis.—That microbic dissociation is
operative in cultures of the organism of Malta or Mediterranean fever
is strongly suggested by certain recent work of Et. Burnet.[85]   It is a
well known fact that many typical, clinical cases of this disease yield
blood cultures with which the agglutination test is negative or slight.
Burnet states that fully one-quarter of all cases fall within this class.
Often the organism obtained agglutinates only in its homologous serum
and sometimes agglutination is quite absent.   As a result of examining
many cultures Burnet points out that nearly all strains of melitensis,
when tested against a type serum, divide themselves sharply into two
groups: those which agglutinate well (titer, 1,000-2,000); those which
agglutinate poorly (titer, 100-200) or not at all.   In some instances there
are "intermediates," but these are few in number.   These circumstances
hold for cultures obtained either from natural infections in goats or from
human cases.   Some serums from goats or human cases agglutinate the
second type of culture only.   Out of 35 strains examined by Burnet, 14
belonged to the second group.   On the basis of these observations this
investigator sets up two distinct types of the Malta fever organism; and
these he refers to as melitensis I and II.   He points out that type II is
identical with the so-called para-melitensis of various workers, notably
of Bassett-Smith;[39] also with organisms of the "group 6" of Evans, and
the "group 4" of Fusier and Meyer.[192] Certain "intermediate" forms are
also mentioned.

* Since the present work went to press there has appeared (November) the splendid antigenic
analysis of the capsulated (S) and noncapsulated (R) forms of Friedländer's bacillus by
Julianelle.[505, 506]   There is not now opportunity of considering in suitable detail the interesting
bearing of this paper on the serologic aspects of dissociation among the capsulated bacteria.
It may only be remarked that the results seem to indicate a correlation between the presence
of capsules and the antigenic basis of the specific soluble substances of the pneumobacillus;
also to indicate that the S type organisms, deprived of their capsules by the acid method of
Porges, behave antigenically like the R forms.   The author believes that the sort of reactions
observed are analogous to those noted by Smith and Reagh and others in the case of their
"flagellar" and "somatic" antigens.   He believes, furthermore, that the dissociation observed
in the pneumobacillus is due to "a cleavage of the specific antigen complex in the animal body
rather than a condition of cultural development."   Although this conception is somewhat at
variance with the conclusions that I have drawn from earlier studies, Julianelle's work intro-
duces certain new problems of considerable interest in the field of dissociative behavior.

When rabbits were immunized with melitensis I and II respectively, serum immune to melitensis I agglutinated I well and II poorly. On the other hand, serum immune to melitensis II agglutinated II well, but I not at all. It is of interest to compare these results with the classical case of Bordet and Sleeswyk [64] in their study of the modified cultures of B. pertussis, as well as with some of the other instances mentioned in this section dealing with the chief culture dissociates, S and R, or in some cases, O. It is also of interest that Burnet states the rabbits given the type II culture were difficult to immunize, but that the agglutination of type II strains usually reached a higher titer than the type I strains. The results of the absorption tests presented by Burnet agree in all respects with the results of direct agglutination. In addition, he showed that several strains of B. abortus (which he as well as Evans regarded as identical with M. melitensis) all fell into the type I group; no abortus strains revealed the type II antigen.

Burnet's type II strains, moreover, were found to correspond with the "para-melitensis" cultures of Bassett-Smith.[39] The latter were found by Bassett-Smith to be characterized by a form having longer rods than the "normal" culture type. This para type, morever, was more easily agglutinated by nonspecific serums and, in addition, showed a tendency to undergo spontaneous agglutination. Bassett-Smith also found rabbits difficult to immunize with this strain.

In discussing the appropriateness of the older term, "para-melitensis," Burnet suggests that when used in this sense, the prefix "para" does not accord the same significance as obtains in the case of the terms, paratyphoid or paradysentery. He therefore suggests the use of "para" for bacterial species which are distinguished by differences in pathogenic quality as well as in antigenic function. For species which depart from the "normal" in antigenic function only (as in the case of his Melitensis II) he prefers the use of the numerals, I, II and III, as in the pneumococcus types. He therefore terms his melitensis variant "Melitensis II."

In addition to the data mentioned above, Burnet presents the results of many tests involving the influence of heat on the antigens of melitensis I and II. These observations also, although not adequately sup-ported by data on the cultural and biochemical characteristics of the two types, are sufficient to suggest strongly that Burnet's type II cultures, as well as paramelitensis of many other workers, represent the R form of culture, or at least a culture (such as O) in the course of transformation to the R state. The serologic tests alone are nearly sufficient to warrant

this conclusion.   Unfortunately the subject of differences in virulence
or in immunizing power were not studied and further work in this field
is likely to add other interesting data.

Although the rôle played by microbic dissociation in the phenomena
described above is strongly suggested, the details become more clear in
a second contribution by Burnet dealing with the phenomenon which
he terms "Entraînement" (influence of one culture on another which
association) and which is considered in detail in section 11 of the present
work.

B. proteus.—In 1916 and 1917 Weil and Felix [483] awakened new
interest in B. proteus by reporting certain observations on variation in
connection with their study of its possible rôle in typhus fever.   Today
we can see that these variational phenomena are concerned with disso-
ciative reactions of a fairly typical sort, although they also present some
points of interest not commonly found in the usual reaction.   Briefly,
Weil and Felix reported the splitting of their culture of B. proteus X19
into two chief types to which they gave the terms, H and O.   The H form,
which appeared to be the normal, grew on solid culture media with a
broadly spreading film ("Hauch"), while the O variant grew in isolated
and discrete colonies without spreading ("ohne Hauch").   In aggluti-
nating serum the H form clumped in large loose ("grob") flocculi,
easily dispersed by shaking.   The O form, on the other hand, aggluti-
nated in small, granular ("fein") clumps.   As was shown by Sachs and
Schlossberger in 1918, when the H antigen was heated at 80 to 100 C.
for one hour, flocculent agglutination (in presence of immune serum)
no longer occurred although some fine clumps were observed.   By a
similar treatment the O antigen was not altered; it still agglutinated in
fine clumps.   These antigenic modifications were soon confirmed by
many others.   In view of these results Weil and Felix concluded that
normal proteus cultures possessed two antigenic groups or receptors, H
(heat labile) and O (heat stable), while the O variant had but one—the
heat stable.   Similar antigenic groups were observed in B. typhosus and
paratyphosus in 1918 and 1920 by Weil and Felix ;[484] also in B. typhosus
by Furth [191] in 1922 and in B. enteritidis by Grushka [217] the same year.
Subsequently they were observed in many other species.   All of these
studies, many of them supplemented by data on cultural variation as well
as serologic, have done much to lend new emphasis to the "double antigen
hypothesis" and the "double" as opposed to the "single" antigen type
of organism.   In casting about for the explanation of these reactions,

however, it is unfortunate that most of the workers have fallen back on the old flagellar and somatic antigen hypothesis of Malvoz, rather than on the newer conceptions of bacterial variation embraced by the phenomenon of microbic dissociation.

In 1919 Braun and Schaeffer [66] attempted the production of "artificial" O types of B. proteus by growth on phenol agar and by "starvation" upon an impoverished agar medium. They made use of the proteus X19 strain of Weil and Felix and succeeded in obtaining types which they believed agreed with Weil and Felix's OX19 in serologic tests. Both types maintained their group antigen (O) but lost the individual antigen (H). Felix and Mitzenmacher,[173] however, held that the phenol and the starvation antigens differed from the "natural" O antigen, although Braun and Schaeffer believed that they had disproved this contention and had shown the identity. Whether the starvation antigens actually did differ from the phenol antigens is not so clear, since the matter was not thoroughly tested. A difference seems probable, however, in the light of the distinct difference in the cell types of the two cultures, as amply demonstrated by Braun and Schaeffer.

Relation of Proteus H and O to S and R Types.—In view of the apparent resemblance in several respects between the H and O forms of Weil and Felix and the S and R forms of many other intestinal bacteria as developed by Arkwright, it was of importance to ascertain to what extent, if at all, the analogy holds true. Such a comparative study was made by Arkwright and Goyle [18] in 1924, followed by a more comprehensive treatment of the subject by Goyle [206] alone in 1926. We may first consider the former report. Arkwright and Goyle observed in B. typhosus, B. enteritidis (Goyle) and B. dysenteriae (Arkwright) antigens regarded as analogous to H and O. They stated that the presence of the two agglutinins could be proved by demonstrating that the type of agglutination is changed after heating an emulsion at 100 C. for 10 minutes; and that "the heated emulsion will absorb S agglutinins but not H, and can be used for obtaining a pure agglutinating serum." They also stated that variants could be obtained containing only one antigen which might be "either R (H) or the S (O)," according to their view.

The conclusions of Arkwright and Goyle are in opposition to the trend of much other evidence. They are, for instance, open to criticism on this ground. In the experiments on B. typhosus and B. enteritidis (conducted by Goyle) it is stated that "in order to obtain 'smooth' variants containing as small a share of the 'rough' factor as possible, the normal cultures were grown on nutrient agar to which 1 in 1,000 phenol

had been added. On this medium daily subcultures were made. . . ."
And again, "these 'S' (smooth) variants have not been obtained by
selecting colonies and consequently may not be so pure nor so persistent
as the 'R' (rough) variants." It is thus (in the case of typhoid and
enteritidis, at least) cultures which arose from phenol treatment, and
which were assumed to be of the S type (in the sense that S is the
"normal," as intimated by Arkwright on p. 110 of the same paper) that
the authors found identical with the O form of Weil and Felix. It
should be added that in the case of B. dysenteriae (in the portion of the
study conducted by Arkwright) it was the "normal" culture without
phenol treatment that was assumed to be the S form. It thus appears
that Goyle, in his portion of the work, modified a normal culture (S)
by use of phenol and then compared the result with O. Braun and
Schaeffer had already clearly shown that one way to change H to O in
proteus was to grow on phenol agar.

Throughout the experiments, moreover, much use was made of
"stock" immune serums, the exact nature of whose antigenic basis is
naturally a matter of doubt. Experimental data based upon such a
manner of scientific approach to a problem of this sort are difficult to
accept as furnishing grounds for satisfactory conclusions. It is natural
that they should have been called into question. In the opinion of
White [487] the conclusion of Arkwright and Goyle that the O and H
antigens were essentially those of smoothness and roughness respect-
ively, was without justification. He believed that their conclusions were
based on two unwarranted assumptions: "that R cultures grown on phe-
nol agar would not also become pure O without at the same time becom-
ing smooth;" and "that any O antigen in the rough cultures was either
identical with that of the smooth cultures or was at least demonstrable
by the sera employed." White was fortunately able to show that the
rough variants contained a heat stable antigen peculiar to the R forms.
For this antigen agglutinins were present in serums made with the R
form, but were absent in serums made with the smooth form. The fol-
lowing conclusions of White may be put more or less in his own words.
The smooth culture normally exhibits both H and O antigens, of which
the former is more obvious in the tests with Salmonella. In some freshly
isolated Salmonella cultures, and in those grown on phenol agar, the O
property comes to the fore as in the case of B. dysenteriae. Roughen-
ing is therefore concerned with a fundamental change in the O antigen,
O smooth becomes O rough, substances which are quite distinct. Often
rough cultures show a sufficient amount of "smooth O-antigen" to absorb

all or nearly all of the "smooth O-agglutinin" from a smooth serum. But a truly "smooth" culture has no "rough O-antigen." The absorption test therefore tends to be "one-sided." White stated further that the process of roughening is often associated with the reduction or the total loss of the H antigen and that the change is permanent. In this manner White brought the serologic changes involved in the H → O transformation into closer agreement with those of the S → R change, but left many points still to be clarified.

In 1926 the proteus dissociation was again considered by Goyle [206] independently. And we may note several important modifications from the results and conclusions previously reported with Arkwright. His work involved mainly a very detailed and clearly presented study, by direct agglutination and absorption methods, of the three forms of B. enteritidis and B. typhosus. These three types were first indicated by Arkwright and Goyle, but characterized more fully by Goyle independently.

Smooth Form Normal: Containing two antigens (similar to those of the H culture of Weil and Felix), the heat labile H and the heat stable O.

Smooth Form Variant: Containing ordinarily only a single antigen, O (heat stable), but sometimes also the R, as below.

Rough Variant: Containing mainly the heat stable R antigen but often some of the heat labile, smooth H antigen found in normal smooth cultures and sometimes, according to White, some O antigen.*

The stock cultures employed by Goyle were all normal cultures serologically, and in agglutination formed flocculent clumps. The R variants were produced by plating out old broth cultures. The smooth variant

* For purposes of effecting greater clearness at this point, attention may be called to the relation between the mode of designating a given culture type and the mode of designating the antigenic configuration or constitution of that type. Although we may refer to one of the chief culture forms by a symbol indicating its most strongly revealed, or so-called dominant, antigen, it must be borne in mind that such a culture may contain one or more other antigens which occur here as minor antigens (quantitatively speaking), but which may also occur as dominant antigens in other culture types. Thus the smooth form of culture may be designated by the symbol, S, because the S antigen is dominant in this culture form. But, at least in many bacterial species, and perhaps in all, the smooth culture also contains a larger or smaller fraction of the antigen O; or sometimes a fraction of the antigen R. This circumstance naturally affords the experimental basis for the recognition of the culture type characterized by the "double," or perhaps we might term it the bivalent, antigen. Whether there exists any culture type possessing the smooth antigen (S) alone, cannot at present be stated, but I am not aware that such a type of culture has ever been described. Thus the smooth culture which we designate S may be said to have the antigenic configuration SO; or perhaps in some cases (dependant upon the species concerned, or the stage of development) the configuration SR. On the other hand, when we consider the antigenic configuration of the "pure" intermediate (O), and the "pure" rough (R), we find that these often seem to possess, respectively, a monovalent antigenic constitution, O and R,—thus agreeing with the symbols designating the intermediate and the rough culture types. At the same time, depending on the stage of development of the culture, it seems that the O culture type may sometimes contain a residual fraction of S antigen; and similarly that the R culture type may sometimes contain a residual fraction of O antigen. Whether any single culture may contain all three of the chief antigens we do not know, but under certain conditions of growth, or on aging, it seems possible. The probability, moreover, that these few bivalent combinations of the chief antigenic units (S, O and R), as recognized at present, by no means represent all of the possible antigenic configurations will receive more detailed consideration on a future page.

O, was produced by growth on phenol agar. All these were employed unheated for the production of immune rabbit serum. All the immune serums were tested against all of the antigens by agglutination and absorption methods. Regarding the agglutination tests the type of precipitate is of interest, and may be found to possess significance in the diagnosis of types among other bacterial species involved in dissociative reactions. For this reason I present Goyle's results in some detail.

TABLE 8

Showing the Type of Precipitate Produced in Goyle's Cases of Simple Agglutination Involving B. Enteritidis Serum Immune to Cultures H (Smooth Normal), O (Smooth Variant) and R (Rough Variant) Against Their Respective Antigens and in Cross Tests [*]

(Data selected from Goyle's tables 2 to 7)

Antigen Culture	Agglutination with Antiserums for		
	Normal S	Variant O	Rough R
Normal S.............................	Flocculent-granular	Flocculent-granular	Flocculent
Variant O.............................	Granular	Granular	Granular
Rough R.............................	Flocculent	Flocculent	Flocculent

[*] Analogous results obtained with B. typhosus.

Table 8 indicates clearly the form of the agglutination precipitate determined by the nature of the antigen or of the immune serum employed, according to Goyle. The normal, smooth antigen (S) precipitates in a form which is a mixture of floccular and granular components in accordance with the double antigenic nature of the culture. The smooth variant, which Goyle takes as identical with the O form of Weil and Felix, always gives a granular ("fein") precipitate, incapable of resuspension. Finally, the rough antigen gives, like the normal smooth, a purely flocculent precipitate but without the granular admixture. All of these precipitate-forms have been described frequently for agglutinating or nonagglutinating cultures, but their relation to distinct antigenic states or forms of culture has not commonly been detected. It would be a matter of considerable importance to be able to correlate the form of flocculation with a definite serologic type, as seems to have been done by Goyle for B. enteritidis and B. typhosus.

It is of importance to note, however, that some others, as Jordan,[274] have found the R type culture characterized by granular antigens rather than floccular. Jordan found that a smooth strain serum of B. paratyphosus B agglutinated R antigen in higher dilution than the smooth itself. Also that the precipitate formed was finely granular. Jordan's rough serum did not agglutinate the smooth strains at all, but agglutinated the R antigens in a granular precipitate. A flocculent precipitate

under these conditions was never observed. There thus appears to be a discrepancy between the results of Jordan on paratyphosus and Goyle on enteritidis and typhosus. We may recall, however, that White had shown that rough cultures of Salmonella often contain considerable amounts of O antigen (which gives, according to both White and Goyle, a granular precipitate). It seems possible that Jordan's R cultures may have contained enough of the O antigen to have given the results observed. In any case Jordan's report raises an interesting question relative to the antigenic transition from O to R culture, and one which is in need of further study. His observations are supported by those of Cowan [111] for her R types of streptococcus, by those of Griffith [215] for the R pneumococcus, and by others.

Turning again to the work of Goyle, it is the absorption tests presented by him that possess greater significance in indicating the antigenic constitution of the three culture types already mentioned. While it is impossible to present in a brief statement the results of the many tests necessary to demonstrate the points at issue, the most essential findings may be summarized for B. enteritidis. B. typhosus, it may be noted, gave almost identical results. In order to make the presentation of this matter more concise I have brought together Goyle's more essential data in the following tabulation:

TABLE 9

SHOWING GOYLE'S ESSENTIAL RESULTS ON ABSORPTION TESTS INVOLVING THE THREE TYPES OF B. ENTERITIDIS CULTURE: NORMAL (N OR S), SMOOTH VARIANT (O), AND ROUGH VARIANT (R)*

(Compiled from Goyle's tables 2 to 7 inclusive)

Antigen Culture	Agglutination Titers with Antiserums for		
	Normal S (N)	Variant O	Rough R
Serums Unabsorbed			
N.........	12,800	6,400	12,800
O.........	3,200	800-6,400	0-1,600
R.........	3,200	1,600	6,400-12,800
Serums Absorbed with N Culture			
N.........		0	0
O.........	No data	0	0
R.........		0	6,400
Serums Absorbed with O Culture			
N.........	12,800	3,200	12,800
O.........	0	0	0
R.........	12,800	1,600	6,400
Serums Absorbed with R Culture			
N.........	800	400	100
O.........	1,600-3,200	800	0
R.........	0	0	100

* Results essentially the same for B. typhosus.

The data presented in table 9 indicate by antigenic analysis the existence of the three chief types of enteritidis culture designated as normal smooth (S), smooth variant (O) and rough variant (R). The antigenic constitution of these may be stated as follows, making use of Goyle's terms of reference.

Normal N (or S): Contains N (= S) antigen (heat labile) mainly; also some O (heat stable) but no rough antigen (R).
O type (= O). Contains O antigen (heat stable) mainly, but also some N (= S); less often some R antigen.
Rough type (= R). Contains the R antigen (heat stable) mainly and usually some N (= S); often, according to White, some O antigen.

These results therefore clearly relate the H antigen of Weil and Felix with the normal or S form, and not with the R as first suggested in the earlier publication of Arkwright and Goyle. Moreover, the O type of Weil and Felix is shown not to be related to the S as first stated by Arkwright and Goyle, but to be an independent antigenic type essentially different from both R and S. For this culture type Goyle lets stand the symbol (O) first used by Weil and Felix. To summarize therefore, we have one heat labile antigen, S (H), and two heat stable antigens, O and R. These may enter into various combinations. These combinations typify the culture types, and show a rather orderly manner of appearance, one being lost as another is gained. The order in which the antigens are gained is, moreover, the same as the order of transition from one culture form to another: $S \rightarrow O \rightarrow R \rightarrow S$. The direction of these changes, which was not pointed out or commented upon by Goyle, is a point of considerable interest which will be referred to later.

The establishing of the O antigen of Weil and Felix as a new antigenic type, apart from S and R, raises the question of the nature of the O culture type, characterized by the possession of this (O) antigen in greatest abundance. I may say directly that I believe that it is the antigen of the transitional or intermediate culture type, or at least one of these types. It arises from S and passes into R. Although this conclusion is not yet proved by evidence which we can regard as conclusive, many observations point to its truth. In the first place it may be pointed out that, although culture types may exist lying outside of the S, the R and the intermediates or transitionals, such have not been reported so far as I am aware, unless we regard the filtrable form of bacteria as a distinct culture type. The expectation is, therefore, that any culture that is not S or R is one of the intermediates, which, as we have seen, embrace a considerable range of variability. In the second place, Braun and

Schaeffer have shown that, when proteus cultures are grown on phenol agar, there is produced, after a few generations, a new form of culture which differs from the S (H) in the morphology of the individual cells. They show all the peculiar shapes which have been observed in the recognized transitional types of other bacterial species. We know, moreover, that this form of culture reverts rather easily to the normal H form when returned to nonphenol mediums. This also is characteristic of the transitional as we have seen in several instances. We also know that a more permanent variant than the phenol O can be obtained by growth of proteus on starvation agar; and that a still more permanent variant can be produced under the influence of immune serum. But I doubt that either of these were the "extreme R" form of proteus. Indeed, it is doubtful if the extreme R of this organism was ever observed by Weil and Felix or others of their school, although it may have been observed by Bronislawa Fejgin.[169] It is only recently that Mr. Weaver in our laboratories has obtained, in the dissociation of a proteus culture on phenol agar, a new chromogenic form, differing markedly from both S (H) and O which, though not yet examined fully, may represent the R type of proteus. It is not until this form has been studied serologically in relation to the known H and O types that we shall have the full answer to the problem of proteus dissociation. The evidence supplied by Goyle on typhosus and enteritidis is strongly suggestive, but in such cases we are scarcely justified in drawing final conclusions from analogies. Proteus X19 itself must be further studied.

To the foregoing exposition a further word must be added regarding terms of reference to the proteus dissociates, in view of the fact that, as we now see, Weil and Felix introduced a new antigenic type, the parallel existence of which in enteritidis and typhoid has been demonstrated by Goyle. Since a part of the undesirable complication in the earlier study of Arkwright and Goyle was occasioned by their use of the symbol S (smooth) in reference to cultures grown on phenol agar, and which as I have intimated were undoubtedly not true S but transitional forms, it seems desirable to return to the precedent set earlier by Arkwright himself (and maintained by him in his section of the paper alluded to)—namely, to let S stand for the normal culture type. Furthermore, since Weil and Felix were the first clearly to point out the distinct serologic characteristics of the type which they termed the O, it seems appropriate to continue the use of this symbol in referring to cultures possessing the characteristics of the transitional form. In this

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group, however, we must expect a greater variability than in S or R because of the great instability of the intermediate type. In proteus Weil and Felix have already called attention to the serological differences existing between OX19 and OX2.

Vibrio Comma.—The serologic aspects of the dissociative reaction in relation to the cholera vibrio have been studied recently by Balteanu,[38] largely following the lines established by Weil and Felix for B. proteus and by Goyle for B. typhosus and B. enteritidis. The results concern partly the two culture types regarded by the author as analogous to the H and O forms of proteus—the former characterized by possession of the double antigen, HO, the latter by the single antigen, O. As has been noted, Weil and Felix extended their early study of the antigens of B. proteus to cover Vib. comma and these authors had in 1920 concluded that in this organism the heat-labile antigen (H) was absent, only the heat-stable (O) being present; that is to say, the heated assumed normal culture agglutinated only in fine granules, without evidence of the flocculent precipitates ("grob") characteristiic of the heat labile antigen. H. Brutsaert[76] also reported in 1924 that he could detect only the heat-stable antigen in the cultures that he examined. As Balteanu states, if the view of Weil and Felix, supported by Brutsaert, is correct, and Vib. comma does not contain the heat-labile antigen, then the case is different from that existing in B. proteus; and, to use Balteanu's own words, "the theory of the labile, flagellar and the stable, somatic antigen is clearly limited in its applicability."

Balteanu made use of two cholera immune serums: one, a Lister Institute polyvalent serum which possesses little interest for us because of the uncertain nature of the antigens by means of which it was produced; the other a monovalent serum immune to the socalled Pottevin strain of cholera vibrio, possessing more significance in our analysis of the reactions. Only the latter will therefore be further considered. One Pottevin serum was produced by the injection of antigen heated at 58 C. for 30 minutes; another by the same antigen heated at 100 C. for two hours. Both serums were employed on a living culture, and on a culture heated at 100 C. Both direct agglutination and cross-absorption tests were performed, and the essential results may be summarized as follows.

The receptor complex of V. comma manifests the same sort of differentiation as that observed in B. proteus and in cultures of the typhoid-paratyphoid group. To state the facts more specifically, there are present

in cholera cultures two antigenic substances, heat-labile H and heat-stable O, corresponding to the "flagellar" and "somatic" antigens of Smith and Reagh in B. cholerae suis. The heat-stable component resists heating at 100 C. for two hours or more, while the heat-labile H is destroyed. The agglutination of the H antigen occurs in the form of loose floccules while the O antigen agglutinates in fine granules. Whole cultures (unheated) give a flocculo-granular or mixed precipitate, except at the upper titer limits where the flocculent precipitate is dominant. Balteanu stated, however, that the differences were not so sharp as in the case of B. proteus, and attributed this circumstance to the fact that a monotrichous organism like the cholera vibrio possesses relatively less flagellar substance than bacteria like B. typhosus and B. proteus.

The data presented above are sufficient to suggest the presence of the dissociative reaction in Balteanu's Pottevin strain of the cholera vibrio but confirmatory evidence is derived from his further study (l.c., part 2) of the serologic behavior of certain "variants" which he obtained from various cholera stocks. As I have already noted in other parts of this work, "variants" or "mutants" of the cholera vibrio have often been described, the modified culture usually manifesting itself by differences in morphology, colony form and biochemical reaction. According to Balteanu, Bordet [57] in 1896 reported inagglutinability of the cholera vibrio as a result of animal passage. Baerthlein,[27] moreover, in 1912, Eisenberg [150] in 1912 and Baerthlein [30] again in 1918 described such colony variants. Shousa [515] also in 1924, according to Balteanu, described an R type of the vibrio differing serologically from the normal form. Cantacuzène [90] in 1925 derived an inagglutinable variant of a vibrio obtained from the marine invertebrate, Phascolosoma, by maintaining a normal culture in contact with immunized tissue.

The work of Balteanu, however, affords more concise information regarding the antigenic structure of the cyclogenic variants occurring in various strains of this species. When old cultures in nutrient broth, peptone solution or on agar were plated this investigator observed among the "normal," round, translucent colonies three variants. These were as follows:

Circumvallate, Rugose Colonies: These were small and yellowish and possessed thickened borders; also radially arranged ridges. These colonies could not easily be suspended in salt solution or distilled water and were firmly adherent to the medium. In broth they formed on the surface a thick, wrinkled film which eventually fell to the bottom of the tube leaving the liquid clear. This type was not constant for, after several days, colonies on agar showed

regeneration fringes from which, by subculture, the original, transparent form could be obtained. The same culture type was observed on old agar cultures after they had begun to become dry.

White Ring-Colonies: These were observed on old agar plates poured from old broth cultures of two of the strains (Pfeiffer and Kedah). "The colonies were whitish and semitranslucent. Sometimes they had an opaque center and more translucent margin, resembling the ring forms of Baerthlein." This culture type was inconstant and reverted to the normal on subculture.

Opaque Colonies: These were obtained from the Pottevin strain. They were usually "prominent, opaque colonies, sometimes with a slightly irregular surface. They appeared dense and white by transmitted light, were very firm in consistency and were adherent to the agar. The growth was extremely difficult to emulsify but by careful rubbing up with distilled water a stable suspension was eventually obtained. Such an emulsion was partially precipitated in salt solution (NaCl 0.85 per cent) after 24 hours at room temperature. Heating to 100 C. somewhat increased the stability in the presence of salt." The absence of any R receptor in this culture led Balteanu to believe it was not similar to the "rough" forms described by Arkwright in 1921. From this type there were sometimes obtained "intermediate colonies" which later reverted to the normal form. This "opaque variant" as a rule, however, was constant on agar, although reversion occurred after repeated subculture in broth. Plating cultures that were 10 to 20 days old yielded cultures with translucent margins; and further subculture from these gave the normal type. This was the only variant that was fairly stable on agar.

The morphology of the third variant revealed an organism somewhat smaller than the normal and surrounded by "a thick layer of pink staining material" . . . . "a sort of slimy exudate simulating a capsule." Sometimes the slimy covering enclosed two or more organisms in a common matrix. Motility in this culture type was absent and flagella were lacking. The range of acid agglutination (ascertained by following the technic of Beiniasch) was higher for the variant (5.5×10^{-4} to 4.4×10^{-3}), than for the original culture (1.38×10^{-4} to 1.1×10^{-3}).

In studying the serologic characteristics of this variant Balteanu prepared four immune serums: one against normal culture heated at 58 C. for 30 minutes; one against normal culture heated at 100 C. for two hours; one against the opaque variant heated at 58 C. and the fourth against the opaque variant heated at 100 C. All the agglutination tests were performed in a medium containing only 0.22% NaCl — in which the variant remained fairly stable. The heating at 100 C. was done in a Koch sterilizer.

The results of the serological tests, designed to reveal the serologic and antigenic relationships between the variant and the normal culture, were as follows: The opaque variant behaved like the O form of B. proteus; that is, only the heat-stable O antigen was found — no heat-labile H antigen was detected. The immune serum produced against the variant, however, contained some H agglutinins, thus demonstrating that some H antigen remained in the O culture. Complement-fixation reactions showed that the O form was as efficient a receptor as the normal, as had also been demonstrated in the case of B. proteus.

One other highly interesting feature of the work of Balteanu was the demonstration that the immune serum derived from the heat-labile antigen H was heat-stable; while the immune serum obtained from the

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heat-stable antigen O was heat-labile. This important observation links for the first time serologic aspects of dissociation with the interesting observations made by Joos [272] in 1903, in which he called attention to the inverse relation existing, as regards heat-stability, in the so-called "a" and "β" agglutinogens of B. typhosus and their homologous agglutinins in immune serums. It thus transpires that Joos was working with cultures containing the "double" and the "single" antigen. This subject is treated in greater detail on another page of this work.

The splendid antigenic analysis of V. comma carried out by Balteanu was purely objective and he did not speculate regarding the significance either of his own or of similar findings in other bacterial species, although he was inclined to relate the differences between the H and O antigens to the presence or absence of flagella, and thus to bring his results into apparent relation with the "flagellar" and "somatic" antigenic hypothesis. Indeed he performed certain experiments with "flagellar antigen" which seemed to support this view. Although I have discussed the subject of the flagellar and somatic antigens at some length in connection with the studies of Smith and Reagh on B. cholerae suis, I may say once more at this point that I believe neither in the work of Balteanu nor in earlier investigations is there any real evidence that such a thing as flagellar antigens, as distinct from somatic antigens, actually exist. It is true that the centrifugal methods, as employed by Balteanu and others to secure "pure flagellar antigen," probably do represent a higher concentration of flagellar substance than is present in the "body substance" (centrifugate); but such "flagellar substance" has never to my knowledge been separated from the soluble or extractive substances also present in the same suspensions. I believe this may be a justifiable criticism of the work of Marion Orcutt [377] on flagellar antigens in 1924. It seems to me, therefore, that we are scarcely safe in regarding serological results that seem to indicate the presence of a heat-labile antigen as due to a flagellar fraction. These heat-labile bodies might equally be regarded as representing soluble substances from the bacteria themselves. Furthermore, we do know, particularly from Griffith's study of the relation between the specific soluble substances of the pneumococcus and the S type of that organism, that the S antigen is more likely to be present in this form of culture as a soluble product than in the heat-stable culture. Moreover, as indicated elsewhere in this work, the phenomenon of the "double" and "single" antigen is by no means limited to those bacterial species characterized by the possession of flagella.

Concluding the matter of the relation of microbic dissociation to the serologic reactions observed in V. cholerae, I believe that the subject has been made sufficiently clear through the facts already presented. The socalled "normal" type of V. comma is the S form (the "helle" of Eisenberg, and the H type of Balteanu) and carries the double antigen, just as in the case of B. proteus. It passes over during aging (B. did not force the dissociation by the use of phenol) to the O form, characterized by the single antigen—and perhaps in some cases to the R type. Around the O type are grouped intermediates or transitional forms, the existence of which Balteanu's descriptions make quite clear. Manifestly, however, this writer did not study, nor attempt to produce artificially, the well-stabilized R form of Vib. comma; at least an R form comparable with that obtained by Firtsch while working with the spirillum of Finkler-Prior in 1888. This possible culmination of his work, following the lines of antigenic analysis already laid down by Goyle, is much to be desired. Our knowledge of the antigenic structure of these forms demands study of the clearly recognized R types as well as of the S and the O cultures.

To the foregoing consideration of Vibrio cholerae it may be added that Shousha [515] in 1924 succeeded in recognizing what he believed to be the S and the R forms of culture and pointed out distinct cultural, biochemical and serological differences existing between these types. It is not established, however, that he worked with well stabilized type R cultures.

Conclusions.—In concluding this section it may be noted first that there exists a far-reaching truth in Baerthlein's statement that, among bacteria in general, colony differences are correlated, not only with striking biochemical differences, but also with fundamental serologic and antigenic disparities of considerable importance. And to this we may add that similar striking variations in antigenic constitution may occur even when the colonial and cultural features appear to be almost identical. These differences seem to involve in most cases a simplified antigenic structure of the R type (and also of the O type, when it appears) as compared with the more complex and variable antigenic structure of the S culture. In other words it is more commonly the O and R antigens that are found in apparent purity, while in the S type culture there is a combination—usually of S and O. On the other hand, the culture characterized morphologically as the R (rough) may contain not only R but also S antigen; and, according to White, some O as

well. The nature of the various antigenic configurations produced by these basic antigens, either alone or in certain combinations, is therefore sometimes such as to warrant the use of the terms "double" and "single" antigen cultures as proposed by Bordet and Sleeswyk for B. pertussis in 1910 and as revived by Weil and Felix for their H and O strains of B. proteus X19, although in other cases "double" antigens may be present throughout. The data already presented, however, make it clear that there exist at least two possible combinations of the "double antigen" type: One is the combination of S with O (undoubtedly typified by the H type of Weil and Felix); the other is the combination of S with R, as observed in all those cases where R is undergoing what we have termed "reversion," but what, as we may eventually find, is not a reversion at all. In addition to these, White has demonstrated apparently the OR combination. It thus becomes an important and interesting question as to methods by which these types of double antigen culture may be differentiated. From the experimental data supplied by Schütze on typhoid, paratyphoid and enteritidis, by Arkwright and Goyle on typhoid, enteritidis and dysentery, by Goyle alone on enteritidis and typhoid and by Weil and Felix, Braun and Salomon, Braun and Schaeffer and a host of other workers on proteus, it becomes apparent that, in the configuration supplied both by the SO culture and by the SR culture, we are dealing with a combination between a heat labile antigen (S) and a heat stable antigen (O or R). It would therefore seem that the differentiation might be effected on the basis of the heat stable component; and here possibly, we can observe a difference. As first shown by Weil and Felix, and as confirmed by most of their followers, the O type of proteus X19 is characterized by the "fein" as contrasted with the "grob" flocculation. It is manifestly the "fein" element (O antigen) that gives the slightly granular appearance to flocculating "normal" culture. This point was confirmed by Arkwright and Goyle in fact though not in actual interpretation; and later by Goyle (in both fact and interpretation) for both typhoid and enteritidis. The heat stable R antigen, however, as shown by Arkwright and Goyle and by Goyle alone, also by some others, undergoes flocculent precipitation and in such cases, in combination with antigen S (as in the rough SR cultures, to be mentioned later), does not introduce the mixed, granular element. Such cultures, therefore, according to Goyle give only normal flocculation. In addition, we know that the rough culture type is characterized by spontaneous flocculation, in which it is likely to differ from the pure O. Outside of these means of detect-

ing the nature of the double antigen cultures, SO and SR, there remains
the possibility of plating, which will usually reveal the R type colony
with its characteristic form and structure. The O colony form may
sometimes differ little from the normal S, or it may be quite character-
istic, as already noted in section 6 of this paper.

In all these distinctions we should bear in mind, however, Jordan's [274]
recent report on the R type of B. paratyphosus B giving granular agglu-
tinations. As stated earlier, the details of the O → R transformation are
in need of further study.

The possible relation of these observations to the interesting studies
of Joos [272] on the dual nature of the typhoid antigen, although not alto-
gether clear, is sufficient to demand their mention. Joos postulated, on
the basis of certain elaborate tests involving typhoid immune horse
serum, produced by both heated and unheated antigens, that
these antigens contained two different agglutinogens, α and
β. The former, he believed, was present in living bacteria, but was
easily destroyed by heating. The β agglutinogen was also found in
living bacteria but was more resistant to heat. Injecting the living bac-
teria into animals, therefore, gave rise to α and β agglutinins, while the
injection of heated culture gave rise to the β agglutinin only.

Thus far we can follow Joos with the analogy of the normal typhoid
culture, containing its heat labile S (or Joos α antigen and its heat
stable O antigen, producing upon injection the "double agglutinin"
immune serum, while the O type culture (or Joos β) produces the
"single agglutinin" serum. Beyond this point we lack data for adequate
comparison. Joos goes on to state that the influence of heat on the
agglutinins is the reverse of its influence on the agglutinogens. The α
agglutinin is heat resistant while the β agglutinin loses its activity when
heated. The α agglutinin, moreover, is wholly incapable of uniting with
the β agglutinogen, while the β agglutinin can combine with either the
α or β fractions of the antigen. These results of Joos were confirmed
in a measure by both Kraus and Joachim [287] and by Scheller.[429] The
second phase of his complicated study goes beyond any available data we
possess on the relation of the S, O and R types to the "double antigen"
reaction, except for the following instance. Balteanu [38] in his recent,
excellent antigenic analysis of the cholera vibrio has shown the presence
of the two types of antigen, H and O, the former being heat-labile, the
latter heat-stable. When these antigens were employed for the
immunization of rabbits, serums were obtained whose reactions have
already been noted. But this observed fact is of significance. The

serum immune to the heat-labile antigen was heat stable, while the serum immune to the heat-stable antigen was heat-labile. It is thus suggested from these limited data that the serologic system with which Joos worked involved the presence of the H and O antigens, if not the R. This problem demands further study.

That the issues involved in these serologic disparities between different subcultures from the same pure-line strains possess great significance for pathology and for serologic diagnosis, as first pointed out years ago by Bordet and Sleeswyk [64] for B. pertussis, by Bernhardt [46] for B. diphtheriae and B. typhosus, and as again called attention to more recently by Mellon [325], cannot easily be denied. For the evidence reported makes it clear that the antigenic relationship between the S, the O and the R forms arising from the same pure line may often be less marked than the relation shown between some of these types and unrelated bacterial species, when judged by agglutination, precipitation or complement-fixation tests and results. As indicated on a foregoing page, Schütze [436] was perhaps the first to point out this circumstance. He showed that serums immune to certain Salmonella strains agglutinated, not only the R form of quite different strains of Salmonella, but also the R forms of B. coli and B. dysenteriae. That these cross agglutinations between different species was due to the heat stable antigen mainly was shown by White [487] in 1925. Moreover, in his positive cross agglutination tests between B. typhosus and B. enteritidis, Goyle [18] showed in 1926 that the reaction may occur with S, O or R forms of culture; but that it ordinarily concerns the heat-stable antigens only, and therefore shows most strongly in the O and R types; indeed he concludes that the heat-stable R antigen is common to both bacterial species. These results seem to go far in explaining not only Schütze's cases of "serological cosmopolitanism" of the rough forms, but also other cases of bacterial convergence already noted. They moreover give us a clue to the assumed enteritidis-typhoid transformations of Sobernheim and Seligmann and of van Loghem; to the assumed paratyphoid-typhoid transformations of Baerthlein and of Bachmann and de la Barrera; to the colon-typhoid mutation of Malvoz; to the convergence of the gonococcus strains observed by Torrey and Buckell; to Esther Stearn's case of convergence of water-borne bacteria under the influence of gentian violet; to the serologic heterogeneity of Reimann's and Stryker's pneumococcus R types, as well as to the pneumococcus-streptococcus serologic affiliations described by Yoshioka, the changes noted by Atkin in the old

stock strains of Gordon's meningococci, and the pneumobacillus-colon-aerogenes serological affiliations reported by Julianelle.[505, 506] Although Reimann did not specifically refer to the matter in his paper on pneumo-coccus dissociation, the agglutinative results which he presents for the R type antigen against standard type 1, 2 and 3 pneumococcus serums·afford, so far as they go, an interesting confirmation of this convergence taking place within the species. In all these instances, one of the most interest-ing features thus concerns, not only the loss of type specificity through dissociation, but also the gain of a sort of antigenic heterogeneity in which the antigenic structure of fairly diverse species comes into apparent relation. In this connection, it·may be of interest, in passing, to recall the bacteriophagic cosmopolitanism in which a similar diversified action is observable. It might make an interesting study to ascertain to what extent, if at all, the distribution of bacteriophagic action parallels the "serologic cosmopolitanism" in the case of some of the intestinal bac-terial types. In another paper [227] I have briefly touched upon this point.

All of these observations combine, moreover, in suggesting to us that the S → O → R transformation is not always so simple a thing as the continuous loss of antigenic substance, as some writers would have us believe in the S → R transformation. It involves also the gain of new antigenic substance to replace the old. Here, there exist problems of much serological interest. But, whatever may come out of it, the diversity of the reactions observed in relation to microbic dissociation can leave us only with a stronger impression of the inadequacy of floc-culation and absorption tests, as usually performed, as a means of differential bacteriologic diagnosis—at least aside from a few well known instances in which their worth has been, in a measure at least, proved. I believe that many of the discrepancies in serologic reactions, as at present employed for diagnosis, will find their solution in the results of the dissociative reaction, operative either in the cultures employed for immunization, or in the test cultures themselves.

It is also apparent, however, that these discrepancies will not be fully explained until we embark on a new trend of study in our search for the meaning of the varied serologic types of this and that pathogenic species. The modern business of collecting new serologic types, apply-ing one or another test, and then constructing artificial pigeon-holes for their safe-keeping, really gets us nowhere; it has not advanced in any appreciable degree our knowledge of the intimate nature of these forms. It seems that what we need most at the present moment is to learn how to create these various types. Then we shall know both their ancestry

and their progeny, and all their interrelations. Then, indeed, we may come to understand their position in what we may sometime recognize as the *antigenic cycle*. Then, and then only, I believe, shall we be in a position to take full advantage of what such new knowledge may make possible in the development of more effective methods of serologic diagnosis, and perhaps of immunologic procedure.

10. DISSOCIATION AND IMMUNOLOGIC RESPONSE

Closely related to the serologic and antigenic reactions of the S, the O and the R types is the question of immunologic response which it may be possible to elicit by the injection of the O or the R type culture, as opposed to the "normal" or S type which has undoubtedly been employed in the greater part of immunization practice. Indeed, the question of the relation of the culture type has seldom arisen, except perhaps in the case of anthrax and a few other infections in which an attenuated virus has been employed in prophylaxis. We have already considered the relation between attenuation and the R type with reference to several pathogenic species; and we have seen that, in many of them at least, the type R culture was non-virulent or only slightly virulent. If the quality of virulence is localized in one or more of the distinct culture types it might seem possible that the immunizing power would also be so restricted to a certain culture form. Bearing on the specific immunizing value of inoculation with clearly recognized type R culture, the first evidence of significance was supplied by the work of de Kruif [118-121] in 1921-22.

The Pasteurella Types.—De Kruif [118] had already demonstrated the virulent character of his type D (S) of Bact. lepisepticum and also the harmless nature of his type G (R) culture (section 8). Two rabbits had been given injections intrapleurally 14 days previously with 0.1 and 0.5 cc. respectively of type G culture, and had survived the inoculation. These animals, along with three controls, then received injections with one cc. of 10^{-4} to 10^{-6} dilutions of type D culture. The principals survived the infection while the controls died in four to nine days.

As de Kruif pointed out, it seemed remarkable that such solid immunity should have been produced by a single, small injection of the type G culture. But this calls to mind another instance. Many years ago I [222, 223] was able to demonstrate for a strain (52) of the organism of fowl cholera (B. avisepticus), which is closely related to, or perhaps identical with, de Kruif's organism, a similar remarkable immunizing power. Less than 1×10^{-8} cc. given subcutaneously to rabbits

produced a local abscess.   Even 3.0 cc. of this culture was easily toler-
ated.   Animals so treated were immune to at least 2.0 cc. of a virulent
culture (48) of which 1×10^{-8} cc. was fatal for unprotected rabbits
within 14 to 36 hours.   Culture 48, when killed and used as a vaccine,
showed slight immunizing ability.   The immunizing power of culture
52 was unique, but its mode of action was never explained.

The results obtained by de Kruif have led me to reexamine this
culture which has been maintained on plain infusion agar for the 12
years or more since the tests mentioned.   I find many of the cultural
features now characteristic of the R type as outlined by de Kruif for
Bact. lepisepticum type R.   That de Kruif has given the true explanation
for my earlier results seems highly probable.

As remarkable as were de Kruif's results in producing a solid immun-
ity against fatal infection with virulent cultures of the bacillus of rabbit
septicemia by the injection of living cultures of the avirulent R type,
they were not new in the field of Pasteurella infections.   In 1919, in
Budapest, Manniger [317] obtained somewhat similar, though less striking,
results by the injection in the living state of what we may now recog-
nize as the R form of the closely related B. avisepticus.   The origin
of this avirulent culture has been described in the section dealing
with "dissociation and virulence" and does not need to be repeated
here, since we are now concerned purely with the immunologic
results.   Suffice it to say that the original culture form (S) was
highly virulent for hens, pigeons, rabbits and mice.   The "mutant"
was practically lacking in virulence and produced death only
when injected in tremendous amounts, as for instance, a complete agar
slant in suspension (larger animals).   When gray mice were injected
once or twice with one-half to one oese of the nonvirulent form they
were able to resist 0.000,02 oese of the virulent culture, which circum-
stance actually represented a marked protection, considering the high
virulence of the normal culture.   When pigeons were injected once or
twice intramuscularly with 2 oese, protection resulted; 0.5 to 1.0 oese,
however, did not protect.   When hens, six to eight weeks of age, were
given 1.0 to 2.0 oese considerable resistance resulted.   With the immuni-
zation of rabbits Manniger was not so successful.   But it is to be noted
that his protective doses were administered intraperitoneally, and I was
able to show many years ago that in immunization with an avirulent
strain of the same organism the intraperitoneal route was of slighter
value.   A distinct reaction (abscess and drainage) was necessary before
immunity could be produced.

Manniger manifestly worked with a culture of the R type—or at least one in the course of transformation toward the R form. It was manifestly not the extreme R, since reversion to the virulent type seems to have been easily possible. When taken into consideration with the results obtained by de Kruif, and with my own earlier results, the record is of much interest since it reveals the fact that the immunizing power of the R type of Pasteurella may not be an unusual occurrence, but perhaps the general rule. These results naturally raise the question as to the possible immunizing power of R type cultures of the closely related human form of Pasteurella, B. pestis. So far as I am aware this question has never been studied. It is of interest, however, that by the use of "bacteriophage suspensions" d'Herelle [246] has been able to immunize with much success against both barbone (Pasteurella bovis) and human plague (B. pestis). It seems clear, from d'Herelle's analysis of the mechanism of the protective reactions here involved, that the bacteriophage is not directly concerned, but that the immunity is due to the generation of protective antibodies possessing opsonic significance. The question arises regarding the extent to which the remarkable results reported may be due to the R type antigen present in the bacteriophage suspensions, i. e., the Pasteurella culture after lysis.

Streptococcus.—Further interest in the relation between dissociation and immunologic response attaches to Cowan's [112,113] work with the streptococcus. Among other points she studied the immunizing value of the less virulent R type when injected into mice and demonstrated that definite resistance was produced against virulent S cultures when subsequently administered by another route. When the R injection was given intraperitoneally it tended to produce abscesses which healed by fibrosis. There seemed to exist a correlation between the formation of these abscesses and the production of immunity. The results obtained by Cowan, however, do not reveal such definite protection as that observed by de Kruif in resistance to Bact. lepisepticum. In the first place the R inoculations of Cowan were themselves sometimes fatal, especially when larger amounts of culture were injected. Moreover, the protective value of R streptococcus was not manifested in all cases. Cross protection (i. e., immunization with an R form of one culture and infection with the S form of another culture) gave some evidences of success, but the number of cases are too few to serve as a basis for final conclusions. Discussing the "local" versus the "general" nature of the protection afforded by inoculation of R culture, Cowan concluded

that it was not of the former type. In some cases heat-killed R culture, as well as S, produced resistance against living S culture.

Pneumococcus.—Regarding the immunologic significance of the pneumococcus dissociates little can be said at present. The only workers to consider the matter in relation to the recognized types are Griffith and Amoss, and neither have dealt with the subject of active immunity as was done by de Kruif and Mary Cowan. Griffith [215] immunized rabbits by many repeated injections of his types S and R, some of the rabbits receiving as many as 26 injections of the latter culture. Protection tests with the resulting serums were performed on mice infected with 0.1 cc. of the virulent type 1 S strain whose MLD was 10^{-8} cc. When injections of 0.2 cc. of S serum were administered with the infecting culture protection resulted. Under the same conditions of infection, however, the R antiserum showed no protective power. No tests involving the production of active immunity by injection of R cultures (which showed so strikingly in de Kruif's case with Bact. lepisepticum) were reported.

Amoss [8] studied the protective value of rabbit S (Amoss' C) and R (Amoss' Z) antiserums for mice infected simultaneously with type S culture. The serums were produced by injection of heat-killed antigens. Two-tenths cc. of serum was mixed with varying amounts of the virulent culture and injected intraperitoneally. Of 15 mice which received each type of serum, one died in the group protected with S serum, while all died in the group receiving R serum. All who have studied the point have reported the greater difficulty in building up a strong antiserum against type R. No report is given of attempts to infect mice which have tolerated inoculations with type R culture, so that we are unable as yet to compare results of pneumococcus and streptococcus immunology.

Salmonella.—White [487] has also made some tests of the immunizing power of R type cultures of Salmonella and observed that the injection of rabbits with the "roughest of the rough strains" (showing no flocculation and no absorptive action on flocculation) gave perfect immunization against the more virulent, smooth strains. A rabbit was immunized with the rough "Kral" strain and was given a final immunizing dose of the living organism. Twenty days later the animal was given one-fourth of an agar slant culture of "Sweet" (smooth), while a control animal received one-fortieth of a smooth slant. The control died in two days while the rabbit immunized with R was unaffected. White thus concluded that "the total loss of flocculating power during roughening

does not appear to influence the immunizing value of the strain." But, since White had already shown that "the destruction of flocculating antigen by heat coincides with almost total disappearance of immunizing activity," he concluded that the true immunizing antigens were "thermolabile bodies, quite distinct from the agglutinin-stimulation antigens. . . ." White also called attention to the point that, not only for the R types of Salmonella but for Shiga and Flexner strains as well, the lack of flocculating power in an antigen (White's R antigen gave granular agglutinations) is associated with slight power to invade the tissues, and therefore concluded that "the antigens which flocculate are essentially substances connected with invasive activity, and that the corresponding agglutinins—probably agglutinins as a whole—are essentially anti-invasive agents." As we may see later, this is no doubt partly true, but the situation will be found to involve still other factors.

The Anthrax Vaccines.—A consideration of the relation of dissociation, and particularly of the R type of culture, to immunologic response would not be complete without mention of the Pasteurian method of vaccination against anthrax, which appears to involve this phenomenon. As is well known, Pasteur [383] found it possible to weaken progressively the virulence of active anthrax cultures by growing them at temperatures above optimum (42 C.) for 15 to 20 days (Vaccin I), or for 10 to 12 days (Vaccin II). Chamberland and Roux [94] produced similar attenuation by potassium bichromate, Chauveau by increasing the atmospheric pressure and Arloing by direct sunlight. Pasteur's cultures lost largely the power of spore formation and virulence and produced some degree of resistance when inoculated into animals. Regarding the actual nature of the modification produced in Pasteur's cultures there exist few original data on which to base an opinion since he leaves us with slight record of the colony characteristics of his cultures. We do know, however, from definite experiments performed in subsequent times, and particularly from the work of Preisz,[394] that one effect of growing B. anthracis at high temperatures is such as to produce two new forms of culture—the "white form" (S ?) and the slimy transitional (O?) which at times seems to exist in great abundance. We know, moreover, that these culture types possess characteristics quite different from the original, virulent form. The slimy, transitional type carries a reduced virulence (which, however, easily regenerates) while the "white" culture is often without virulence. Although it can scarcely be doubted that the immunizing value of Pasteur's vaccines is in some way connected with these modified culture types, strange as it may seem, there appear to

be no records as to the behavior of pure cultures of these widely differ-
ent forms in immunologic or serologic reactions. It seems probable,
however, that the Pasteur vaccines are virulent cultures which have been
partially attenuated by heat-modification in the direction of the S form;
and that the degree of this modification is greater in the case of Vaccin I
than in Vaccin II. The ultimate understanding of these matters can be
reached, however, only when more definite experiments have been con-
cluded making use of the clearly recognized forms (R, S, and transi-
tional) of the anthrax bacillus produced in controlled laboratory
dissociations. Mr. Nungester in our own laboratories has definitely iso-
lated these chief types by means of the normal dissociative reaction and
colony selection and has studied them sufficiently to validate many of the
observations of peculiar colony variations presented in earlier pages of
this paper.

The Cholera Vibrio.—Balteanu [38] in his excellent study of the H and
O antigens of this organism isolated a socalled opaque variant, which
was probably the intermediate or O form; also a rugose variant, prob-
ably the R. Although the latter was not studied fully, the normal form
was contrasted with the opaque variant with reference to immunizing
power. The actual tests were, however, somewhat limited, represent-
ing only a comparison of the cross reactions. Twelve guinea-pigs were
given three successive doses. Six received culture of the normal form
and six received culture of the opaque variant. All were tested 10 days
after the last dose. The six receiving the normal form were tested with
the opaque variant, while the six receiving preparatory doses with the
opaque variant were tested with the normal culture. In the words of
the author, "All lived except one, which had been vaccinated with the
original form and this one died 2 days after the test dose of the opaque
variant." Unprepared controls injected with one-half the test dose of
the two forms respectively died in 10 to 12 hours. The author's con-
clusion was that the opaque variant gave "as good protection as the
normal." He had previously shown that the opaque variant was "rather
less virulent." Unfortunately the immunizing power of the form in
which we are at present particularly interested, namely the R, was not
tested. An interesting field of study thus lies open in respect to the
immunologic reactions of the cholera dissociates.

Nature of the Immunologic Response.—To the above one further
point may be added regarding the possible nature of the immunologic
response. In numerous instances as will be shown, (section 11,
Immune Serum), contrary to the mass of textbook information on the

subject, one influence of homologous immune serum upon normal, virulent culture is to accelerate a transformation into the commonly less virulent and more easily phagocytable R type culture. Such observations, involving largely experiments in vitro, but also many somewhat more obscure observations of reactions in vivo, might appear to bring into view a new immunologic principle relative to one aspect of the body defense against virulent, invading bacteria, involving the opsonins and their actual mechanism in promoting phagocytosis. The matter may be stated briefly as follows:

We know that normal serum, unless perchance germicidal for the bacterial species in question, usually has the ability to hold the S form of culture up to type; and perhaps, in some cases, to enforce the R → S reversion. We also know that homologous immune serum in suitable strength has, on the contrary, the ability (in vitro, and to some extent certainly in vivo) to effect a transformation from S to R. We know, moreover, that immune serum contains antibodies in the form of opsonins or bacteriotropins and that these often have the marked ability of facilitating phagocytosis both in vitro and in vivo, by reason of a still unexplained influence on the bacteria.*

Finally, we know that, in certain cases at least (pneumococcus, Pasteurella, pneumobacillus, for example), the R type of cell is easily and quickly phagocytosed in vivo while the S type is not.

The interesting question therefore arises: Are the immune bodies that we call opsonins and bacteriotropins agents by whose action the transformation in type of the infecting organism in immune serum (in vivo) is accomplished? In other words, is the "preparing action" (on the bacteria), commonly attributed to the opsonins, merely the enforcing upon the virulent forms of a dissociation by virtue of which reaction they become amenable to phagocytosis? I may say in conclusion that many details of the opsonic theory of immunity (which is surely convincing in its broader aspects) are not so clearly established but that renewed study might be profitably undertaken along the lines of this conception. In such a case we might be led to consider with greater interest than heretofore the obscure "antiblastic theory" of immunity clearly suggested by Charrin and Roger [96] in 1892 and elaborated by Ascoli [20] in 1908 in opposition to Bail's [31] aggressin theory. But in this connection, if the transformation from S to R can be demonstrated to occur in vivo as readily as in vitro, then these two at present opposed

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* Bull [80] has expressed the view that the agglutinins play a part in the protective reaction.

theories will be brought into unity through their mutual relation to the dissociative reaction. I believe that a conception of immunity based upon such a unification of the antiblastic and aggressin hypotheses, and embracing our present notions of the trophic antibodies, may yield the most effective basis for new lines of immunological study lying outside of the toxin-antitoxin reactions.

Finally I wish to call attention to one further point of immunological significance dealing with active and passive immunity in two species in whose mode of action in infections there are many similarities—Bact. lepisepticum and the pneumococcus. De Kruif [119] has pointed out for the R type of the former (as I also noted [223] many years ago for an unrecognized type of the Pasteurella, B. avisepticus*) a most remarkable immunizing power through the agency of living R type cultures. He did not test the protective value of the serum immune to the avirulent R type. On the other hand, Griffith, and after him Amoss, showed the high degree of protection resulting from the use of serum immune to the S type of pneumococcus and the absence of protective power in the R immune serum. They did not test the immunizing action of injections with living R type culture. In view, however, of the results of de Kruif (and it may be added of Cowan on streptococcus immunity, and of P. B. White on immunity from an R type Salmonella) the important question arises : In the case of the pneumococcus can a durable immunity be produced by the injection of living R type cultures, comparable in effectiveness with the passive immunity afforded by the injection of S immune serum? Notwithstanding the important results already reported on pneumococcus dissociation, these immunologic aspects of the problem have been omitted by Stryker and Griffith as well as by Reimann and Amoss. Also in the case of Bact. lepisepticum we still await information regarding the presumable lack of protective power in the serum immune to the R type. But in all the results that are thus far clear we find the inherent suggestion that there may exist in the body a type of immunity whose potency is not appreciably manifested by antibody phenomena, and which is not transferable by means of socalled immune serums. It may be that interesting results

* In 1914 I wrote (l. c., p. 401) the following: "May we regard this as a hint that in other pathogenic species there exist, among the many avirulent strains, one or two perhaps that may be characterized as immunizing cultures? . . . One might inquire whether, among the many avirulent cultures of the pneumococcus . . . there exist strains which, by some form of inoculation or through some strictly local reaction, would call forth the immune response to more virulent culture material. . . . In this disease . . . might it not be worth while to make a systematic study of the reciprocal relations existing between various virulent and avirulent types?"

will come from a further study of the immunizing properties of certain R type cultures of pathogenic bacterial species.*

Conclusion.—In the section dealing with the relation of dissociation to virulence it has been shown that in one and the same bacterial culture there may exist, side by side, cells or colonies, some of which represent organisms of the highest virulence while others represent organisms possessing no virulence whatever. From the data presented in this section it begins to appear in a similar manner that the diversity of biologic activity manifested by different cells in the same culture may be carried even further. In one and the same bacterial culture there may exist side by side cells or colonies some of which represent organisms that are of no or of slight value for immunizing purposes; others which represent organisms of remarkable immunizing power but destitute of virulence. In 1914 I [223] pointed out that certain cultures were not necessarily of immunizing value merely because they were virulent or otherwise "typical." Apparently this conception is not limited to different cultures occurring independently in nature, as I then assumed, but may even concern different organisms in the same culture, and even in the same pure-line. I believe that these later observations open a field of considerable significance for immunological theory and practice. There now may certainly be said to exist, not only in nature but also at times in the laboratory, cultures of pathogenic bacteria that may properly be spoken of as "immunizing cultures," because they possess this power far in excess of other cultures of the same species. They are not ordinarily, I believe, to be found associated with acute disease; nor are they to be found commonly in pure form in laboratory stocks. But the point of special interest is that such cultures can be produced artificially by resort to appropriate methods of cultivation. Some of these methods are already known and others will undoubtedly be discovered. It is needless to say that they may not be the same for all bacterial species (section 11).

As to the extent of applicability of this conception—that must be left for the future to ascertain. At present we can safely say, however, that the fact is established for Bact. lepisepticum and B. avisepticus

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* In quite recent studies on the immunologic aspects of the dissociative reaction in Friedländer's bacillus (pneumobacillus) Julianelle [505, 506] has reported some facts of interest. Serum immune to the capsulated S type (and therefore containing the type specific immune bodies) was found to protect white mice against the virulent S culture. Serum immune to the noncapsulated R type (and therefore lacking the type specific immune bodies) had no protective influence in 0.2 cc. amounts. This immune serum likewise failed to agglutinate the capsulated cells or to precipitate the soluble specific substance. In a similar manner serum immune to the R nucleo-protein (acetic acid precipitable material) failed to protect when given in similar amounts. The protective value of immunizations by means of the R cultures was not reported, and therefore this question still remains open for the pneumococcus.

among the Pasteurella, and will probably be shown for B. pestis, another
member of the same group. Also, though to a lesser degree, for cer-
tain strains of the hemolytic and the greening streptococci; and probably
for Salmonella. The situation with reference to the pneumococcus
remains to be ascertained. In this case and in many others the arti-
ficial production of cyclogenic variants, and a careful comparison of the
relative immunizing power of these dissociates, is at present one of the
most important fields of investigation in vaccine therapy. It may not
be found that it is always the R type of culture that is of chief immun-
izing significance (and the situation may not be found the same with
toxic forms as with those characterized by marked aggressiveness) any
more than it may always be observed that it is the S type culture that
carries virulence. Different bacterial species may vary in this regard.
The important fact to bear in mind, however, is that we are coming to
regard not only virulence, but also immunizing power, as a quality not
distributed equally through the elements of the culture, but localized in
certain cells existing in a definite, but doubtlessly transitory, cyclogenic
stage.

11. THE INCITANTS TO ACTIVE MICROBIC DISSOCIATION

Of the cases of microbic dissociation thus far mentioned perhaps the
majority have been observed as occurring "spontaneously" or have been
the incidental or accidental result of endeavors having another purpose
in view. In the majority of instances, moreover, the striking phe-
nomena observed have not been recognized at the time as possessing
significance other than that they were "mutations," or that they demon-
strated the possibility of adaptation to a new environment, or that they
manifested the nature of "impressed variations." Major interest has
nearly always centered in the end result of the process and seldom in
the nature of the process or reaction itself. Indeed, if any investigator
has considered the process at all, it has usually been in terms of some
simple sort of mechanism by virtue of which certain pre-formed bac-
teria, better fitted to survive, did survive; while others, unfitted, per-
ished. It was as simple a reaction as the "survival of the fittest"—and
one possessing about the same general significance. For this reason
microbic dissociation has not been studied as a significant, independent
biologic phenomenon and few experimental attempts have been made,
either to prevent its appearance or to enforce it when it might be of
service; moreover parallel dissociative trends among bacteria at large
have seldom been noted.

The earliest methods consciously employed for securing that sort of microbic variation now termed dissociation involved the use of old broth or agar cultures, or old gelatin cultures as employed by Firtsch [178] in 1888, and emphasized anew by Baerthlein [30] in his study of colony variation and correlated features in 1918. Baerthlein plated on agar, incubated for 24 hours, then permitted the plates to stand at room temperature for some days or weeks. Similar methods had been employed by Preisz for B. anthracis, by Feiler for B. proteus, by Eisenberg for many species and by Penfold and others for intestinal bacteria. Under these conditions daughter colonies of a new type often arose within, or at the margin of, the old colonies. These new colonies represented the "mutant" type and could be isolated in pure culture. In other cases Baerthlein merely plated old broth cultures and obtained directly two or more colony types with their characteristic culture growths. Arkwright, Weil, Felix, Eisenberg, Wreschner, Manniger and many others have also employed this method.

Although in the case of many bacterial species one merely needs to be on the watch for dissociation to make its appearance in old cultures on solid mediums, or in old broth tubes, there can be no doubt that dissociations similar to those described in previous pages can be produced in the laboratory easily and quickly as soon as we have discovered the critical environmental conditions required to incite the process; or to develop it to macroscopically observable proportions.* At least I believe we are justified in searching first for the "critical environment," rather than for things more intangible and mysterious.

That the critical environment or condition will be the same for all species of bacteria seems unlikely, although that all conditions might impress the physiologic mechanism of the organisms in a somewhat similar manner can scarcely be doubted. Different substances are required for growth of different bacteria, but they all grow; different substances may possess different lethal or bacteriostatic effects for different bacteria, but death or bacteriostasis results in all cases. Similarly, I believe, we may be justified in assuming that different incitants to dissociation may be required for different bacterial species, although the same one might be sufficiently effective for several members of the same bacterial group, and perhaps for members of some other groups as well.

* For reasons which will subsequently appear, I believe it doubtful that, in "enforcing dissociation" on a culture, we impress upon it any reaction that is foreign to its established physiology. "Enforcing a dissociation" is more probably bringing into the foreground, and making observable macroscopically, a type of physiological behavior already inherent, and presumably obscurely operative, in the culture mass. We must recall that normal, smooth cultures always contain some O antigen.

With the exceptions to be mentioned later, we have little concise information regarding the influence of various incitants to dissociation. On the strength of numerous incidental observations, however, it is suggested that the category of incitants may include such diverse stimuli as operate through temperature, food substances, physical state of the medium, volume of the medium, oxygen tension, desiccation, antiseptics, foreign protein, products of bacterial metabolism, body secretions, normal serum, specific antibodies, products of growth (including microbic associations) ; and to these may perhaps be added the influence of certain colony types.   Certain of the more conspicuous instances in which some of these incitants to dissociation are found to be operative will be reviewed in the following pages.

Temperature.—That temperatures higher than the optimum are able to stimulate variation in cultures has long been known; and practical use has often been made of the fact. It was observed clearly in pigment production (Laurent [297]) although here the phenomenon may concern only fluctuating variations. Also in fermentative reactions (Wilson,[488] Coplans, Adami, according to Gurney-Dixon [229]) and in virulence, as early exemplified in the first immunization experiments of Pasteur with fowl cholera and anthrax, and in many other cases in more recent years. In Pasteur's work with anthrax protection it scarcely need be said that his cultures, modified by growth at 42.5 C., were relatively nonvirulent in proper doses and were ordinarily nonsporogenic. This was demonstrated with special clearness by the studies of Pasteur, Chamberland and Roux [383] in 1881. Bail [32] later demonstrated the effect of heat in modifying the abilty to form capsules and the closely related character of virulence. Preisz [394] showed the effect of heat in causing the development of peculiar mucoid colonies of the intermediate or transitional type between the S and the R forms. The studies of Katzu,[276] also on anthrax, have again quite recently revealed the influence of high temperatures in producing peculiar secondary colonies. In all of these instances we can recognize the modification of the culture toward a "mutant" form of growth.

The transformatory effect of temperatures higher than normal on virulent clinical types of the diphtheria bacillus was first recorded by Roux and Yersin [416] in 1890. The results clearly dealt with the production of O and R forms which were nonvirulent. Cultivation of virulent cultures at 39.5 to 40 C. yielded nonvirulent cultures resembling the pseudodiphtheria bacillus and characterized by different colony formation, modified growth in broth, changed cell morphology and fermenta-

tion features. There can be little doubt that Roux and Yersin produced the dissociation of the diphtheria bacillus by heat. Hewlett and Knight [251] manifestly accomplished the same result seven years later by heating their virulent cultures at 45 C. for 17 hours. With reference to the colon-typhoid-dysentery group, it is scarcely necessary to mention the long list of workers who, beginning with Rodet in 1894, have observed heat derived variations, some of which were manifestly what we now regard as the R type. Further, we have the dissociation of B. proteus X19 from the H into the O form under the influence of heat (42 C.) as shown by Weil and Felix, Hirschfeld and Zajdel [252] and others. Similar results were obtained by Braun and Schaeffer [66] by the use of phenol agar and by "starvation" agar. The derived O forms were apparently the same serologically, and were similar to the "natural" O type of Weil and Felix, although further study of this subject is needed. Hirschfeld and Zajdel, also Sachs and Schlossberger,[420] studied the same problem in B. typhosus, B. paratyphosus B, and B. dysenteriae.

Heat has also been employed for the purpose of producing serological variations in pneumococcus and streptococcus. In 1922 Yoshioka [494] reported obtaining from virulent pneumococcus cultures of the three standard types serological and cultural variants as a result of growing in successive passages at a temperature of 39 C. Similar results were obtained with Aronson's virulent streptococcus. When we compare the sort of variants thus obtained with those produced by Griffith in 1923 and by Reimann and Amoss in 1925 under the influence of immune serum, it is clear that Yoshioka's variants represented the R types, or transitional forms moving in this direction of variation.

In the section dealing with "dissociation and virulence" I have mentioned the curious instance reported by Reddish [402] from Rettger's laboratory dealing with the socalled B. sporogenes which was believed by Reddish to be a necessary contaminant in nearly all of his botulinus cultures from various sources. I have also suggested that this form might have been an R type of the normal B. botulinus, produced by the action of heat as commonly employed by American workers in preparing the cultural material for dilution and plating in the process of isolation. If later work should demonstrate this to be the case, here also we would have another instance of the influence of heating on the production of dissociation in an anaerobic species.

Quite recently Soule [450] has shown the influence of temperatures higher than optimal in forcing the dissociation of B. subtilis. Whereas in broth at 26 and 28 C. the reaction occurred slowly, raising the tem-

perature to 37 C. greatly accelerated the reaction; and when the broth
cultures were grown at 45 C. a large percentage of dissociation occurred
within a few days. When agar slant cultures, which ordinarily showed
slight or no dissociation in the earlier hours of growth at room tempera-
ture, were maintained at 37 C. dissociation progressed rapidly.

Food Substances.—In this connection the work of Reiner Müller,
Thaysen and of Penfold are of special interest. Penfold [386] in 1912
reported a "mutational" change in colonies of bacteria of the intestinal
group characterized by the formation of "papillated colonies" within the
mother colonies after a period of five to nine days when the organisms
were grown on agar containing various sugars; or, in one instance,
sodium mono-chlor-acetate. The daughter colonies, similar to those
seen earlier by Neisser, Massini, R. Müller and others, were regarded
as mutants, although the process was not spoken of as dissociation. Per-
haps the clearest case among those reported by Penfold is that of B.
typhosus seeded on isodulcite agar. Here the daughter colonies appeared
red, giving indication of fermenting the isodulcite while the original
culture was unable to do so. It may be added that these results were
obtained with cultures arising from single cell isolations. The new form
was spoken of as "an isodulcite mutant" and occurred uniformly in the
case of all typhoid strains studied. Reiner Müller [357] had already
shown in 1909 a similar mutational capacity on isodulcite, and in addi-
tion that B. coli colonies did not respond in this manner.

Similar results were obtained by Penfold by growing B. typhosus on
lactose agar plates containing neutral red. He was able to demonstrate
also "raffinose mutations" in the case of B. paratyphosus B, but not in
the Aertryckè bacillus. This difference Penfold regarded as the only
cultural distinction and one worthy of use as a common test. In addi-
tion he demonstrated the influence of sodium mono-chlor-acetate in
causing many intestinal bacteria to form papillated colonies. The
"mutants" in this case were more resistant to the medium, but were in
turn able to give rise to other papillated colonies when the strength of
the sodium acetate was further increased. This reaction was observed
in B. coli, B. enteritidis, B. paratyphosus A and B, and in B. grundthal,
but not in B. typhosus. Six "sucrose mutants" were mentioned by
Thaysen [460] in 1911.

Eisenberg [149] has also shown the readiness with which the anthrax
bacillus could be transformed from the spore to the sporeless state by
growth on glycerol agar. This result Eisenberg attributed to the acidity
produced from glycerol fermentation, although others assigned it to the

special germicidal influence of glycerin. If acid substances stimulate this change, the case is at variance with most instances in which it appears that dissociation occurs most readily in an alkaline medium. Glucose mediums produced similar though less uniform results. De Kruif [120] has reported that high concentrations of peptone favored dissociation in Bact. lepisepticum.

Wilson [488] in 1906 obtained, through the addition of urea to culture mediums, results in culture modification which we may regard as incipient dissociation. From B. typhosus, B. coli, B. pyocyaneus, B. enteritidis and Friedländer's pneumobacillus he thus produced filamentous and leptothrix forms. Adami, Abbott and Nicholson [1] observed similar effects from the addition of saliva to medium in which B. coli was grown. According to Gurney-Dixon [220] Connal [108] has shown that the spinal fluid, in cases of spinal meningitis, may contain as much as 0.5 % of urea, and it is thus interesting to consider the relation of such an urea content upon the dissociation of the meningococcus in vitro, as also of the influenza bacillus in the spinal fluid as reported by Ritchie [406] in 1910 in cases examined by him.

Under the heading of the influence of food substances (although the circumstance probably involves osmotic and other influences) the study of Matzuschita [321] in 1900 on the effect of high salt content may be mentioned. Matzuschita grew various species on agar medium containing from 5 to 10% of sodium chloride. The organisms are reported to have assumed unusual forms and to have given modified cultural features. Cocci gave off rod or filament forms. In addition, many rod forms produced, besides long filaments, simple cocci and giant coccoid bodies such as have been described numerous times in cultures manifestly undergoing the early stages of the dissociative reaction.

In concluding this subject a word may be added regarding the effect of sewage in producing dissociative changes in many species of bacteria. According to Wilson (1910) (See Gurney-Dixon [220]) Almquist noted that the effect of sewage on B. typhosus was the production of leptothrix forms, such as are observed at the beginning of the dissociative reaction in many species of bacteria. The effect of sewage in producing mucoid forms of culture has been noted since by many investigators. Almost any sample of sewage when plated on Endo agar reveals many socalled mutants of intestinal bacteria, especially of B. coli. Similar results have been obtained from the application of sewage filtrates to pure cultures of B. typhosus and related organisms. In addition, it is now a well-

known fact that sewage filtrates represent the most prolific source of lytic principle, active against many species of bacteria. From sewage-contaminated river (Huron) water I have isolated lytic principle for all of the following bacterial species: Bact. aerogenes, B. alkaligenes, B. cavisepticus, B. coli, B. cholerae suis, B. dysenteriae, B. enteritidis, Bact. gallinarum, B. icteroides, Bact. ozenae, B. paratyphosus A and B, Bact. pneumoniae, B. psittacosis, Bact. pullorum, B. pyocyaneus, Bact. rhinoscleromatis, B. typhosus, B. typhi murium, M. albus. Students working in my laboratory have obtained lytic principle for M. citreus, Streptococcus fecalis and Streptococcus lacticus. Also Koser[285] has obtained one for a true thermophile. Sewage filtrates are thus highly provocative not only of microbic dissociation, but also of transmissible autolysis.

Starvation.—"Starvation methods" have also been employed for the purpose of enforcing dissociation. These are exemplified by the work of Braun and Schaeffer[66] on Proteus X19 and of Feiler[167] on the typhoid bacillus. Braun and Schaeffer employed an agar medium containing a minimum quantity of nutrient broth and observed that after a few transfers on this medium the X19 strain was transformed to one giving the cultural and serologic characteristics of the OX19 of Weil and Felix, a type which we can recognize as possessing some characteristics of the R, and which corresponded also with the form obtained from phenol agar ("single antigen" type). Following the method of Braun and Schaeffer, Feiler in 1921 produced a similar transformation in the typhoid bacillus. The "permanence" of the starvation culture was less marked than that of the phenol dissociate; and much less than that of the dissociate produced by the action of homologous immune serum. The types of Proteus X19 produced by starvation were found by Braun and Schaeffer to have lost all motility and all flagella. Feiler found the starvation R type of B. typhosus composed mainly of short plump forms with no motility. According to his observations phenol worked a more thorough dissociation in respect to producing nonflagellated forms. Moreover, the reversion to the normal type of culture on normal mediums was effected most quickly with the starvation dissociates, more slowly with phenol cultures, and most slowly with the R types produced by immune serum.

Physical State of the Medium.—There exists universal agreement among all investigators who have studied the dissociation problem, knowingly or unknowingly, that a liquid medium favors or accelerates

the reaction, while a solid medium delays it. This circumstance was pointed out early by Penfold and later (with full recognition of the dissociative process) by Arkwright,[16] de Kruif [120] and by Webster.[481] Soule [450] has more recently found that, while the type S subtilis is highly stable on agar, the dissociation proceeds rapidly in cultures maintained in plain broth. I have observed the same phenomenon in many cultures of members of the colon-typhoid-dysentery group. Even the presence of an undue amount of water of syneresis at the bottom of an agar slant culture may be an important factor in many cultures. Eisler and Silberstern [156] were thus able to show that cultivation of B. typhosus on moist and dry agar gave respectively two antigenically different types of culture. On the other hand, rapid transfers through plain broth may apparently increase the S type or even cause a reversion of "pure" R strains (Jordan [273]). Feiler [167] found that such rapid passage of an immune serum R strain of B. typhosus effected a transformation to the S type after some 18 passages; while 25 passages on slant agar determined no change in the R culture. Apparently a dry medium is best calculated to produce the greatest stability in both R and S cultures, and such observations are confirmatory of the older observation of Beijerinck [42] to the effect that microbic stability is always most marked on solid mediums. This view was also upheld by Eisenberg in all of his many and varied studies on bacterial variation.

When we turn to the effect of liquid culture mediums, more specifically upon some of the curious intermediate (O) types, we observe a particularly unfavorable action on the stability of the culture so far as continued dissociation is concerned. Here we note the rapid disappearance of the O type as narrated by Firtsch [178] for Vibrio proteus, the disappearing O form of B. typhosus in broth, as depicted by Bernhardt,[49] and the rapid disappearance of certain presumably intermediate types of B. coli in broth as described by Bordet [61] in connection with his studies on transmissible autolysis. Many other observers have noted colony forms of various bacterial species which would grow on agar, though perhaps faintly, but not in broth. It can scarcely be doubted that the dissociative reaction is the explanation of many of these instances. As we shall see later, these curious phenomena may have a close bearing upon the significance of the bacteriophage and its mode of action.

Volume of the Medium.—Certain experimental data, particularly those of Soule [450] on dissociation in B. subtilis, have shown that dissociation does not occur under conditions in which there are no opportunities for growth, as in physiologic salt solution for example; or in a tube of

broth medium when the active stage of growth has been passed. It is thus suggested that if two different volumes of medium, one small the other large, were employed, the degree of dissociation, after a given time, would be greater in the large volume than in the small one. Such a test was actually performed by Soule, the amounts being about 5 cc. of broth in a tube and about 300 cc. in a flask. Each was seeded very lightly with B. subtilis type S. After 16 to 18 hours active growth in the tube ceased and the degree of dissociation, as ascertained by plating tests, had remained at a low point. In the 300 cc. flask, however, growth was still proceeding actively after 60 hours and the percentage of dissociation had at this point reached a much higher value. We may thus conclude that, if the medium is one in which dissociation can occur at all, the results will be more pronounced as the volume of medium is increased.

In this connection it is of interest to note that the volume of the medium employed may also have an influence on the time required for the reversion of the R type to the S. This point which was studied by Feiler [167] is considered in the following section. From these facts one might predict that a procedure for raising virulence by increasing the relative number of type S organisms would succeed as well through the inoculation of a single large volume of medium as by repeated transfers through several small volumes.

Oxygen.—Few data are found bearing on this point. Novy and Soule [499] observed that when the available oxygen surrounding agar slant cultures of B. malleus was reduced to 0.1% (but in large volumes of gas) peculiar erosive changes in the culture, strongly suggestive of dissociation took place; and that these were accompanied by the generation of a new form of culture growth quite different from the original. These changes have not been fully studied. On the other hand, it can be observed that daughter colonies of B. typhosus, as reported by Morishima [352] (four strains), appeared within three to five days under anaerobic conditions while, under aerobic conditions, they required eight days. Webster [481] has stated that the dissociation of Bact. lepisepticum is concerned with the oxygen relations and can be prevented in broth cultures by the addition of hemoglobin, serum or certain inorganic catalysts. Soule has thus far not been able to detect an appreciable influence of modifications in oxygen content upon dissociation in B. subtilis although some of his experiments suggested the influence of 10% CO_2 (in either air or nitrogen) in maintaining or "protecting" the S type against dissociation on agar plates. In this instance the

criterion was the "marginal" dissociation which often affects colonies that have started as S type culture. In connection with the reversion of the R form of M. tetragenus (noncapsulated) to the S type (capsulated) Wreschner [492] has reported that, in tubes of serum-broth exposed to air, a 25% dissocation occurred in 24 hours, while in similar tubes in which the medium was covered with paraffin, no reversion whatever had occurred within the same period. This is in accord with my own experience that secondary R colony formation is less common in agar slant tubes that are rubber capped or sealed with wax (after a brief period of growth in air) than in tubes left open to the air permanently. We know, however, from the study of Schattenfroh and Grassburger [427] that the dissociation of the butyric acid bacillus (anaerobic) takes place under anaerobic conditions of growth. In this case, however, the cultures do not grow well under aerobic conditions. The relation of oxygen to the inciting of dissociation is thus in need of further study. It may be pointed out, however, that there is some evidence to indicate that the R type culture grows best under at least partial anaerobic conditions; it is more commonly found at or toward the bottom of the tube. Firsch [178] in 1888 clearly showed the difference in the top and bottom areas of gelatin tubes as concerned the distribution of the chief types of Vibrio proteus. These and similar observations raise the question regarding the nature of the modified, nonvirulent culture of B. tuberculosis obtained by Vaudremer [472] in deep, nonglycerolated potato culture. The manner of growth and the morphology of the cells, taken in conjunction with the absence of virulence, is suggestive of the R type of culture in general, although of the fact of dissociation in B. tuberculosis we at present know little.

Antiseptic Substances.—The influence of antiseptics in producing dissociations is detectable in many older contributions to the subject of bacterial variation and mutation. Phenol was the first of these substances commonly employed. In 1890 Chamberland and Roux [94] reported the influence of phenol in modifying the virulence of B. anthracis. In this case a different type of culture was produced. Phenol, among other means, was employed by Malvoz in 1892 in his alleged (Villinger [475]) transformation of B. coli into B. typhosus, "or near to it." In this case he grew his colon cultures in phenol broth at 42 C., and it is therefore difficult to differentiate between the effect of the phenol and the effect of the heating. Unquestionably, however, this treatment served to produce R types of B. coli, which, as we now know from the work of Schütze,[436] may show analogies with other members of the

colon-typhoid-dysentery group and paratyphoid-enteritidis group as well. Indeed, we may perhaps recognize in the report of Malvoz one of the earliest instances dealing with what we now term "bacterial convergence." Villinger [475] in 1894 studied again the influence of phenol on B. coli and although recognizing its modifying effect on cell morphology and motility, as well as on other characteristics, could not agree with Malvoz to the extent of believing that a transmutation to B. typhosus had occurred. A good review of many of these old experiments dealing with the colon-typhoid transformation is to be found in the work of Kiessling [277] in 1893. Fraenkel [183] also in 1889 in his study of the effect of phenol on bacteria has presented data bearing on the dissociation problem.

In studies on B. proteus X19 phenol has often been used to enforce the dissociation from the H type to O, which as a result of the studies of Arkwright and Goyle,[18] Whitè,[487] and Goyle [206] alone, we have come to regard as an intermediate form. Braun and Salomon,[67] as later Braun and Schaeffer,[66] employed phenol agar (0.17%) for the production of their OX19. Feiler [167] accomplished a similar dissociation of B. typhosus by a similar means. In the early work of Arkwright and Goyle on B. enteritidis, B. typhosus and B. dysenteriae these workers at first believed that they had increased the S fraction of mixed S-R cultures by the use of phenol. This result, however, was quite opposed to those of Feiler's detailed and careful experiments and cannot be accepted in the light of much contradictory evidence. Indeed, Goyle has come to a quite different conclusion in his later paper. This point has been considered further in section 9 of this work. In addition to the above instances, Jeanne Lommel [511] has most recently reported the influence of phenol and of formol as modifying the fermentative characteristics of B. coli, resulting in the production of a strain that fermented saccharose and thus resembled B. communior.

Regarding other antiseptic substances, Penfold [385, 386] has shown the influence of sodium mono-chlor-acetate in enforcing dissociations of B. typhosus, and causing the formation of secondary cultures. F. M. Burnet,[87] while observing the S → R transformation of B. typhosus in plain broth, also noted the higher proportion of R forms produced in oxylate broth. Eisenberg [148] showed the origin of secondary colonies of B. typhosus when cultures were submitted to the action of egg albumen, which Rettger has regarded as possessing germicidal power for several bacterial species. Chamberland and Roux [94] showed the partial dissociation, accompanied by loss of spore formation, of B.

anthracis when cultures were submitted to the action of a 1 : 2,000 dilution of potassium bichromate. Möhler and Washburn, according to Gurney-Dixon,[220] produced variants of B. diphtheriae by the use of iodin terchloride.

The action of antiseptic substances has also been studied with reference to producing in chromogenic bacteria changes which are presumably on the order of dissociations although certain aspects of the matter are not clear. Among these contributions we find of particular interest those of Franz Wolf[490] (1909) and of Baerthlein[28] (1912). Wolf studied B. prodigiosus, Sarcina lutea and Staphylococcus albus and aureus. Serial growth of B. prodigiosus in mediums containing traces of potassium permanganate, cadmium nitrate, mercuric chloride or potassium bichromate produced some dark red races and some white. Such changes often seemed to be permanent. It is of special interest that the same antiseptics (for example, mercuric chloride, cadmium nitrate and potassium permanganate) could produce both dark red and also white races. The dark red forms produced in this way were permanent but the white often reverted. Dobell[130] believed that these changes were purely a matter of chance and might be "permanent, partly permanent or impairment." In a few instances Wolf observed white strains, simulating M. albus, arising from M. aureus.

Baerthlein plated out old broth or agar cultures of B. prodigiosus and obtained some dark red colonies and some white. Others were white with red spots, or red with white sectors showing the so-called "sector-mutation." According to Baerthlein these "mutant races" bred true in most cases but might show reversions if left for a considerable time in the same culture tube.

Dyes.—The forcing of dissociation by dyes has received considerable attention under the guise of securing the adaptation of bacteria to antiseptic or bacteriostatic agents. Thus Ainley-Walker and Murray[478] in 1904 noted that the action of methyl violet on B. typhosus, B. coli and the cholera vibrio caused the development of variants. Also, in 1906 Loeffler[307] reported that by the use of malachite green he had produced four new types of B. coli which remained constant in their new characteristics. Two of these we can easily recognize as R types. In 1912 and 1913 Cecil Revis[404] studied the adaptation of B. coli to malachite green and to brilliant green. He found the culture, through "training," could be made to grow in 0.05% of brilliant green but in plates containing this amount two types of colony arose. The two forms differed in cultural features and in fermentation reactions, and undoubtedly represented the S and R type.

Referring again to B. coli, a report by Esther Stearn [452] on adaptation phenomena in some water-borne bacteria is of special interest in showing certain dissociative changes. This investigator was able to demonstrate successive biochemical alterations in B. coli-communis A and B, B. aerogenes B and in the lactic acid bacillus, under the influence of gentian violet (1 : 200,000). The degree of change varied with the time of exposure (48 hours, 120 hours, 5 months) but interest centers especially in the fact that, whatever the organisms were at the start, all of them came through the experiment at the end of five months manifesting the type reactions of B. coli communior A. Even the lactic acid bacillus ultimately reached this endpoint. It seems probable that the results in this case were due to the dissociative reaction, determined by the dye and by aging. If this is so they possess the significance of demonstrating that the changes which occurred were cumulative over a considerable time, although some of the cultures showed change at the end of 120 hours. So long as it is able, the organism apparently responds to meet the changing exigencies of its environment. Here we apparently have a case of bacterial convergence as indicated by the fermentative reactions of the endforms, which may be regarded as the R types of the respective cultures employed, and we may bear in mind the serologic convergence of the R forms of Schütze (typhosus, paratyphosus, dysenteriae and coli) ; also the apparent serologic convergence of the gonococcus cultures whose "common type" was pointed out by Torrey and Buckell. If the work of Esther Stearn can be confirmed, the observations will mark one of the significant findings in the field of dissociation and bacterial convergence. It seems probable, however, that serologic tests of the end forms might have revealed differences not shown in the fermentation reactions.

The foregoing observations for the most part made no mention of the nature of the phenomena involved except for the occasional statement that they concerned "mutations." The characteristics of the mutating types were seldom followed up, and most of the descriptions of them are meagre. So far as I am aware, the only investigators up to 1926 to report actual experimental studies involving in any way the conditions determining dissociation (with the exception of those concerned with the H and O forms of proteus, in which dissociation was naturally not recognized) were Cowan, de Kruif, Webster, Griffith, Reimann and Amoss. Cowan worked with the streptococcus, de Kruif and Webster both with Bact. lepisepticum; Griffith, Reimann and Amoss with the pneumococcus. Other dissociations have been reported as such, but the interest has centered in the results rather than in the process.

Even Cowan's work at the beginning was not so much a matter of enforced dissociation as the taking advantage of slow and natural variations of cultures on blood agar, whose value in affording R cultures she estimated by testing the ability of certain colonies to yield spontaneous agglutinating growth (indicative of the R type) in broth. It is by no means necessary, however, to perform such a tedious selection in order to produce dissociation in the streptococci, for variants often arise on blood agar plates when seeded with old cultures. There is little doubt that a still more rapid dissociation of streptococcus can be incited by some such simple means as those employed by Stryker, Yoshioka, Griffith, Reimann and by Amoss, to be considered subsequently; or such as employed by Atkin (deep, Gordon "trypagar" plates grown for a few weeks) for colony dissociation of the gonococcus and the meningococcus. Faith Hadley [499] has secured striking dissociation in colonies of Streptococcus fecalis, mitis, salivarius and hemolyticus by cultivation for several weeks (first at 37 C., then at room temperature) on deep "trypagar." Under these conditions the colonies may often attain a diameter of 12 to 18 mm. in the case of fecalis, and reveal a great wealth of daughter-colony and other structural variation. It is only by studying microscopically as well as macroscopically the remarkable features of such giant colonies that one can properly appreciate the undoubted complexity of morphologic and physiologic behavior occurring within an isolated streptococcus colony. To conclude this phase of the subject, however, it may be said that probably any bacterial species may reveal marked colony variations provided it can be made to grow for a sufficiently long time on solid mediums. The chief reason why such phenomena have not been observed more frequently in the past is that colonies seldom have time to mature before the medium dries and growth is suspended. Dissociation, we must not forget, is dependent on continued growth.

Reaction of Medium.—In Bact. lepisepticum de Kruif [120] has shown dissociation in cultures freshly isolated from rabbits. The reaction occurred rapidly in broth and peptone solutions but more slowly on agar and only to a slight extent in mediums containing normal horse or rabbit blood or serum. An acid medium (P_H 6) retarded the tendency while an alkaline reaction (8.5) often accelerated it. Attention may here be called to the fact that Gratia [209] pointed out that 8.5 was most favorable for transmissible autolysis, and that d'Herelle [248] recommends 7.8. Both Reimann [405] and Amoss [8] reported 7.8 as the most favorable reaction for pneumococcus dissociation, while acid mediums hindered the reaction. The older observations of Preisz [393] involving what we can now

certainly recognize as dissociation in B. anthracis were made on cultures
grown on "alkaline agar." In dissociations of many members of the
colon-typhoid-dysentery group, also in the capsulated bacteria, I have
found that a reaction of P_H 7.8 favors the dissociation. Apparently B.
pyocyaneus does not dissociate in an acid medium.

Animal Passage.—Although animal passage has always been recog-
nized as the most effective means of exalting the virulence of patho-
genic bacteria it has seldom been employed for the recognized purpose
of modifying the morphologic or cultural type. It was employed unsuc-
cessfully by Eisenberg [149] in 1912 in the attempt to cause a reversion
of a nonsporogenic race of B. anthracis to the sporogenic type. Many
passages through guinea-pigs showed the culture to possess some resid-
ual virulence but its nonsporogenic character was not changed. Preisz
had already stated that when the sporogenic function had once been lost
it could not be regained. Bail and Flaumenhaft,[34] however, who obtained
the two colony variants, presumably S and R, of which only the lat-
ter (their β form) was virulent, found that the inoculation of guinea-
pigs with R gave typical anthrax death and only R organisms could be
found on autopsy. The inoculation of the S type (their α) resulted in
a greatly delayed death; and then also only the R form could be
obtained at necropsy. From examinations made at intervals before the
S-inoculated animals died, they noted that the R form began to arise
at about the 90th hour of the infection and increased from that time
onward.

Baerthlein [30] in 1918 showed that passage through normal animals
caused the heightened development of the S type (capsulated) of the
pneumobacillus from mixed S and R cultures; but that, when the
"pure" R form (noncapsulated) was injected, it did not revert to the S
state. The same thing was shown by Wreschner [492] for his "absolute"
noncapsulated variant (R) of M. tetragenus when passed through mice.
The influence of passage in stimulating the overgrowth of the S form
of Bact. lepisepticum was demonstrated by de Kruif; also by Mary
Cowan for streptococcus and by Griffith, Reimann and by Amoss for
the pneumococcus. Curiously, Schmitz [434] obtained different results in
his injections of guinea-pigs with pure line strains of B. diphtheriae. By
intraperitoneal injections, as well as by the use of collodion sacs, he was
able to demonstrate that, in the course of time, the virulent form was
robbed of all of its chief characteristics and became nonvirulent. The
effect of passage of S cultures through immunized animals usually
gives this result, so far as present data indicate. Whether the S → R

transformation occurs under natural conditions in the living animal as soon as the bacteriotropic antibodies have adequately developed is a highly interesting and important point because of its bearing upon a possible mechanism of body defense to which attention has already been called by Griffith with reference to pneumococcus infection, and which has been considered in detail earlier in this work. Griffith was unable to detect evidence of the S → R transformation in the living animal (immunized) although the S form of the pneumococcus, after being injected, rapidly disappeared from the body. It is logical to expect that further study will reveal this transformation occurring in vivo.

With respect to the effect of passage of virulent culture through the normal animal Yoshioka [494] has recently obtained modified, avirulent forms of the virulent, hemolytic, Aronson streptococcus by following the method of Morgenroth. A mouse was given injections with the virulent type. Four hours after the injection a culture was taken from the subcutis and plated. Among the mass of hemolytic colonies could be found a few of the greening variety. These were nonvirulent and gave quite different serologic reactions. Naturally these methods involving animal inoculation for the production of variants are open to some criticism, but support for their significance is added by the circumstance that the modified culture was serologically similar to the dissociate produced by growing the virulent type at 39 C., and by other methods. Unfortunately, however, Yoshioka did not present data on the correlated cultural features of the variants, a matter which always lends valuable support to any conclusion regarding such assumed modifications, so far as the production of the R form is concerned.

Regarding the influence of passage of virulent cultures through immunized animals, several instances bearing on this point are presented in the following pages dealing with the influence of continued growth in immune serum. We shall see that the most common effect is the production of a modified culture which has lost all, or at least the greater part, of its original virulence.

Normal Serum and Ascitic Fluid.—Although it has often been recognized that normal blood produces marked changes in the form of bacteria, whether these changes are always of the nature of dissociations is not so clear. Observations of this sort begin to appear in the literature soon after the description by Pfeiffer of the phenomenon which bears his name, and have concerned largely the typhoid bacillus and the cholera vibrio. Regarding the latter there is evidence that, not only immune serum but also normal serum, may cause a granulation of the vibrio

and that at least some of the granules are viable when transplanted into
a fresh medium. This has been demonstrated more convincingly, how-
ever, for the action of normal serum on the typhoid bacillus for which
many normal serums are known to be germicidal. Knowledge of these
matters was first presented by the various works of Pfeiffer, Kolle and
Fränkel, and Radziewsky;[400] and further by the work of Eisenberg[147]
who presents an excellent review of the influence of both normal and
immune serum up to the time of his own work in 1903. It would seem,
however, that a dissociative influence of normal serum is not clearly
established for B. typhosus unless it also possesses some natural ger-
micidal power. Danysz[115] showed that when "first vaccine" anthrax
culture was grown in rat serum the organisms were modified. Since,
however, the first vaccine is itself a modified culture, the final results
obtained by Danysz will not be further considered. Hess[250] in 1921
showed that when virulent anthrax cultures were grown continuously
in normal horse serum (12 hour passages) they lost their capsules in
from 14 to 31 generations. The loss was permanent unless the organ-
isms were again grown in animals. It occurred more quickly at 41 C.
than at 37 C. It is of interest that one of the cultures (Stamm III) with
which Hess worked possessed the characteristics of what we have
termed the S type. This was transformed to a noncapsulated state in
the shortest time of all—namely, seven generations on normal serum.
Many other workers have produced modifications of cultures by growth in
normal serum, but the changes reported are not such that we can ascer-
tain that the transformation to the extreme, or even to an intermediate
R form, was attendant. In the case of most pathogenic organisms the
result has been, rather, an increase in the S type. When the foreign
serum has intrinsic germicidal influence the result is manifestly dif-
ferent.

To the above it should be added that aging an S type culture in
contact with normal serum (as in a 10% serum-broth) may not result in
producing an increase in the number of S organisms in the culture, and
in this respect is quite different from actual passage of organisms in
series through serum-broth cultures. Nungester,[499] for instance, has
observed that aging a culture of B. anthracis in such a medium for some
weeks resulted in an increase of R type organisms. The same reaction
was observed not only in the case of the S type culture, but also in cul-
tures of several other types (including the slimy variety) which Nun-
gester succeeded in isolating from his original pure line of B. anthracis.
The R type culture seemed to be the common goal of all the transform-

ations. Similar results were obtained by aging in plain broth. Results from serial passage through serum-broth are not yet available from Nungester's experiments.

The modifying effect of normal serum on bacteria submitted to its action in the course of culture has to do, in part at least, with the phenomenon (often mentioned in the literature) of "serum-fastness," as treated by Braun and Feiler,[65] Rosenthal[413] and others. Such an adaptation to normal serum is manifestly accompanied by a modification of the organisms in which they become more resistant to whatever slight germicidal power the normal serum possesses. Rosenthal showed that such serumfastness of B. typhosus to normal rabbit serum occurred after three or four passages. Such resistance of the bacteria was not correlated with increased resistance to chemotherapeutic agents and was not "type-specific." It was still detectable after 20 passages in broth without serum. On agar the reversion to normal was accomplished more quickly—sometimes after a single passage. It is perhaps important to note that such cultures possessing maximum fastness for normal serum showed almost normal agglutinability, which indicates that they had undergone no significant beginning of the transformation toward the R type. The strains grown in inactive serum, on the other hand, showed, together with normal susceptibility (i. e., no serumfastness), a strong agglutinin-fastness. It is also important to note that "fastness" to the bactericidal power of the serums did not lend to avirulent bacteria the quality of virulence. With reference to this point Rosenthal states: "Die Festigkeit gegen die bakteriziden Substanzen des Kaninchenserums verleiht somit den Typhus Bazillen nicht die Eigenschaft der Virulenz." A similar result was obtained by Braun and Feiler. Although the accomplishment of a "fastness" to bactericidal influences did not serve to increase virulence, Rosenthal regarded such an adaptation as a necessary preliminary to the establishment of an infection.

The dissociative influence of ascitic fluid in culture mediums is well known to all who have worked with problems of mutation in pathogenic forms; and it may be that a part of the aversion to its use is due to its influence in effecting variations of such magnitude that they have been regarded as contaminations and assigned to the ascitic fluid as a source. The dissociative action of peritoneal fluid is to be seen in the reference by Crowell[114] to the transformation of the virulent, granular type (S) of the diphtheria bacillus into a nonvirulent, solidly staining coccoid form in the peritoneal cavity of the guinea-pig. In the case of normal serum, whether the organisms are killed or permitted to grow in a modi-

fied form, seems to depend on the strength of the serum, much as we shall see is also the case in immune serum. The modified type has usually been reported as a nonagglutinating culture.

Immune Serum and Immune Blood.—The use of normal blood or of blood serum for increasing virulence is too well known to demand further consideration; and the manner of its action in accomplishing such an effect has been shown in the experiments of de Kruif, Cowan, Griffith, Reimann and Amoss, to mention only more recent studies, as well as suggested by the reports of numerous earlier writers (Eisenberg, bibliography). The influence of immune serum, however, demands further attention. A survey of the effects of such serum on the "normal," virulent type (in the cases to be reported it seems justifiable to assume the S type or largely so) or on the definitely recognized S type, seems to reveal something of a discrepancy. Some works indicate the transformation of the virulent form into one possessing little or no virulence, and often characterized by a spontaneously agglutinating or non-agglutinating (in immune serum) manner of growth. Other reports, on the other hand, indicate a heightening of the virulent character by much the same method, indicating a sort of immunization of the bacteria against the destructive working of the immune serum. This last conception is theoretically plausible; and, on the strength of a few questionable instances combined with certain speculations of Welch, has descended through some generations of textbooks to such an extent that it may be said to represent at the present time the most common view of the matter. Zinsser (p. 249) [496] for instance states, "This lessened susceptibility to antibodies is noticeable not only in strains cultivated from the body in disease, but can be produced artificially by cultivating bacteria in inactivated homologous immune serum." After citing the observations of Walker [477] on B. typhosus and of P. Th. Müller [355] on B. typhosus and Vibrio comma, both of which indicated increased virulence of the respective organisms in immune serum (and both of which have been amply contradicted by much later evidence), Zinsser says, "Such strains not only increase in virulence but lose in both agglutinability and susceptibility to bactericidal effects." Again he states (p. 13), "but additional evidence pointing in this direction has been brought by experiments in which it was shown that bacteria cultivated in the serum of immune animals not only gained in resistance to destruction by the serum constituents, but at the same time were rendered more pathogenic." And again (p. 13), "It is interesting, moreover, to look upon this process of adaptation as a sort of immunization

of the bacteria against the defensive powers of the host, a conception early suggested by Welch." In the following considerations we shall see that a view of the influence of immune serum in producing non-agglutinating forms of bacteria is amply justified by an abundance of evidence; but that this phenomenon is accompanied, as a rule, by greater virulence, as most commonly reported in the textbooks, is a conception against which a great mass of evidence is directed. In much of this evidence we shall see that many instances support the view that the fundamental phenomenon determining the observed changes is microbic dissociation.

Metchnikoff [345] in 1887 reported that the virulence of B. anthracis was diminished when the organisms were brought into contact with anthrax immune serum. Anthrax organisms which developed in the blood of sheep that had undergone vaccination lost the power of killing rabbits. Roger,[409, 410] also Charrin and Roger,[95] demonstrated the same phenomenon when they injected virulent streptococci, pneumococci and B. pyocyaneus into rabbits that had been immunized with these organisms, respectively. Indeed these authors [96] in 1892 formulated a theory of immunity based on the observed attenuating power of immune blood, according to which virulent organisms were not killed but lost their pathogenic power when coming into contact with the blood of immune or vaccinated animals. In these cases we unfortunately know little regarding the cultural or serologic modifications impressed upon the organisms concerned.

In 1893 Sanarelli [422] studied the influence of serum immune to Vibrio metchnikovi against the homologous organism. He inoculated virulent cultures into animals that had been vaccinated, and found that, although the bacteria had not been killed in the body, they had lost their virulence and were noninfective. He ascertained, moreover, that the same result appeared when serum from the vaccinated animals was brought into contact with the vibrios in vitro. In these cases, however, the inoculum containing the assumedly attenuated microbes also contained some of the immune serum. When the organisms were removed from this serum by subculturing in broth or by washing, and then injected, Sanarelli found that they possessed as much virulence as the original unmodified culture; sometimes more. He therefore came to the conclusion that the influence of the immune serum was, not to produce an attenuation, but sometimes even to increase virulence. In interpreting these results, however, we must bear in mind that the vibrio was not cultivated for any length of time in the immune serum

(only 48 hours), and we are not aware that the immune serum possessed any unusual strength. We are not therefore justified in assuming from the results of these tests of Sanarelli, or others reported by Metchnikoff [347] in 1892 on attenuation of the hog cholera bacillus, that the growth of pathogenic bacteria in immune serum had the effect of increasing their virulence, although this has been the conclusion usually drawn from these cases, as well shown by the comment of Stryker [454] on these and similar instances. We shall be able a little later to compare these results with others in which we can be assured that the bacteria have actually been grown (continuously) in immune serum. And we shall see moreover why growing bacteria in immune serum for 24 hours, or even 96 hours, may not be sufficient to eliminate their virulent features when they are again placed on a normal culture medium.

Hamburger [231] in 1903 demonstrated that the cholera vibrio gained virulence when cultivated in normal guinea-pig serum but lost it and appeared as another type of organism when cultivated in guinea-pig cholera antiserum. Another report bearing on the same organism is that of Ransom and Kitashima.[401a]

Nicolle [366] in 1898 demonstrated the influence of typhoid immune serum in transforming the normal type into a self-agglutinating form, clearly the R type, but which was still virulent. The sojourn of the culture in the serum was, however, brief and immediate reversion occurred. Saquépeé [421] in 1901 accomplished somewhat similar results by growing B. typhosus in collodion sacs in immune rats. After a period of five months the culture became nonagglutinative, simulating the so-called "eberthiformis" type obtained from natural sources. In vitro the results were not so clear. Typhoid culture was placed in a tube containing 5 drops of broth together with 15 drops of immune serum. At the end of 45 days no change in the agglutinative power had occurred. When, however, the medium was changed every few days, a slight reduction in agglutinability occurred. The question of virulence was not considered. Steinhardt [453] in 1904 reported that when grown in strong immune serum typhoid and dysentery bacteria lost their virulence and became spontaneously agglutinative, then yielding cultures which we can now recognize from her description of the colony form as belonging to the R type. Somewhat similar results were reported by Reiner Müller [358] in 1911. Moon [351] in the same year showed that two distinct types of B. typhosus arose from a single cell—one agglutinable, the other not. Zinnser and Dwyer, according to Morishima [352] made observations in 1918 on the loss of agglutination by B. typhosus. Especially

convincing studies, however, so far as their bearing on dissociation was concerned, were reported by Feiler [167] in 1920; others by Morishima in 1921.

Feiler studied in considerable detail the influence of phenol, starvation and immune serums on strains of B. typhosus, and B. paratyphosus B, following the same general lines as those of Braun and Schaeffer for B. proteus. By all the means mentioned Feiler was able to produce from normal, motile cultures variations characterized by modified cultural and serologic features; also by loss of motility and flagella. While phenol and starvation, according to Feiler's view, caused a loss of certain agglutinogens, continued cultivation in homologous immune serum caused the loss of all agglutinogens. Feiler thus held that the loss of agglutinability involved the loss of receptors—partial in the case of phenol but complete in the case of typhoid immune serum. He also held to the distinction between ectoplasmic (flagellar) and endoplasmic (somatic) agglutinogens, as earlier treated by Malvoz, Nicolle, Smith and Reagh and others. The permanence of the changes produced was also discussed, as considered further in section 12 of the present work. Feiler's results agree fairly well with the earlier reports of H. Sachs [419] and of Weil,[482] Felix and Mitzenmacher [173] on B. proteus; and he accepts a similar explanation, based on the "double" and "single" antigen (receptor) hypothesis. He did not present data on the virulence of the modified types.

The work of Morishima in 1921 containing data on the influence of immune serum on B. typhosus followed the trend of Feiler's study. Since this has already been mentioned in the preceding section, and since one important aspect will be referred to later in the present section, it is sufficient to say here that, like Feiler and others, Morishima was able to produce a different and nonagglutinating form of the typhoid organism by serial cultivation in typhoid immune serum. One may perhaps conclude from his results that the organisms passed through the intermediate (O) stage, in which they were inagglutinable, to the R form in which they were again agglutinable, although the report lacks data on the cultural features of the variants. In general Morishima's results confirm the results of Feiler's more detailed and comprehensive presentation of the serologic aspects.

Regarding the diphtheria bacillus, we have no exception to the general rule that a strong immune serum is able to effect a transformation of the virulent S type into the nonvirulent R form. Although this has been accomplished by several workers, the clearest picture is given by

Bernhardt [49] in 1915 in his valuable study of the various diphtheria types. He made use of immune human and guinea-pig serums, both active and inactive. To the immune serum in one cc. amounts he added varying quantities of 24 hour virulent culture and sampled by plating at 24, 48 and 72 hours. From such plates he obtained atypical colonies giving short rods of the Hofmann type (diphtheroid), which for the most part were nonvirulent. His further description of these forms indicates that they were an R type of the diphtheria bacillus as outlined in a previous section, but probably not well stabilized.

With reference to the pneumococcus, many older reports have indicated a transformation in the type of culture from a virulent to a nonvirulent by the use of immune serum. The latter type usually gave a spontaneous agglutination and was noncapsulated. Older observations dealing with this point are found in the works of Friel, Laura Stryker and Yoshioka. Much more conclusive experiments on the variation in this species, and with full recognition of the chief dissociative types, S and R, have been reported in more recent time by Griffith, Reimann and Amoss. Jacobson and Falk [266] have also reported obtaining rough colonies by growing cultures in broth containing immune serum. These reports will be mentioned presently. First we may review the older studies.

Friel [185] in 1915 demonstrated that when virulent cultures were grown in homologous immune serum, they underwent definite cultural changes, lost their virulence and became phagocytable even in normal rabbit serum. Laura Stryker [454] in 1916 reported growing virulent pneumococci for many generations in a broth with 10% homologous, immune serum. She observed that in all cases (types 1, 2 and 3) a new form of culture was produced lacking both capsules and virulence. The original culture was modified by degrees but eventually came to resemble what we now recognize (mainly through the work of Griffith) as the R type pneumococcus. The essential characteristics of Stryker's modified types have been presented in detail in section 9. The modified strains remained permanent even when grown on normal mediums without immune serum, but reverted in all cases after several passages through mice. Stryker believed that the degree of permanence was correlated with the number of passages through immune serum broth and the truth of this view was shown later by Griffith. The work of Yoshioka [494] in 1923 was largely confirmatory of the significant results presented by Stryker seven years earlier. He obtained similar modifications of his types 1, 2 and 3 pneumococcus cultures by drying and

also by growing at 39 C. The variant culture showed itself by loss of virulence, decrease in agglutinability in immune (homologous) serum, and increase of agglutinability in heterologous sera, thus indicating bacterial convergence. These changes did not appear at the same time in all of the cells of the culture but only in certain colonies. The detailed serologic aspects of Yoshioka's work have been considered in section 9.

Since Griffith [215] (1923) is the leader in this field of study on the pneumococcus in which attention was focused on the R and S types in particular, his results are worthy of special notice. This is also true because he worked with standard type 1, 2 and 3 pneumococcus cultures. Virulent cultures (type S) were grown in series in concentrated homologous immune serum, heterologous immune serum and normal serum being used as controls. While at the beginning the cultures commonly showed only S colonies, after the first immune serum transfer some R colonies appeared on plating. The second serum transfer yielded a larger number of R colonies and, after the third transfer, R culture had entirely replaced S. These results, obtained with concentrated immune serum, could be duplicated in some measure by the use of immune serum in a dilution as high as 1 : 256. Heterologous and normal serums generally had no power to produce R colonies; once a type 2 antiserum gave R colonies in a type 1 culture. Griffith reported one experiment that is almost unique in dissociation studies: he tested the dissociation-provoking power of R and S antiserums on S type culture. A virulent S culture was grown in R antiserum; three passages gave no change in the organisms concerned. On the contrary, when S culture was grown for one generation in the S antiserum, a mixture of S and R colonies resulted. A colony of each type was picked from these plates and the subsequent cultures injected into mice. The S culture was fatal in a dilution of 10^{-7} cc. while the R culture was harmless in 0.2 cc. amounts.

The only instance, so far as I am aware, in which both S and R immune serum has been used against both R and S forms of culture, is found in the work of Soule [450] on B. subtilis. Soule made use of these immune (rabbit) serums in various dilutions but found that 10% was as effective in accomplishing the results reported as any higher concentration. When R and S forms of culture were placed in the S immune serum, no change occurred in R, but the S culture gave 80% dissociation into R at the end of 24 hours. This was the most complete and rapid dissociation observed by Soule under any conditions. When, on the other hand, R and S cultures were placed in R immune

serum, the S type culture underwent no appreciable change, while the R culture experienced a 40 per cent reversion to the S type. So far as I am aware, this is the only instance reported indicating the reversion-producing effect of an R immune serum on R culture. It naturally opens several interesting lines of inquiry.

To the instances mentioned above may be added a reference to the tubercle bacillus, although we know little regarding dissociation in this species. Karwacki [508] has recently observed that by cultivating the tubercle bacillus in the blood serum obtained from tubercular cases he could obtain a morphologic dissociation of the inoculated organism. There arose in the culture coccus or rod forms that lacked the usual acidfastness. The colonies of these nonacidfast organisms grew more rapidly and became larger than the normal colonies containing the typical acidfast bacilli. Although many important details are lacking in this study, the presence of the dissociative reaction is strongly suggested. Apparently the tubercle bacillus is no exception to the general rule that growth of a culture in immune serum of immunized or partially immunized animals, is one of the surest methods of initiating those cultural changes which concern the dissociative reaction.*

Although the majority of evidence, as indicated briefly above, demonstrates the fact that virulent bacteria, grown continuously in immune serum, lose their agglutinative reaction and usually their virulence, and sometimes become spontaneously agglutinative—all of which suggests a change to the R type of culture—there are on the contrary some instances in which a heightened virulence and no appreciable change in the type of culture have been reported. These include the observations on B. typhosus by Eisenberg; [147] also Steinhardt [453] found that in weak immune serum the virulence of dysentery and typhoid cultures was increased and that the S type of culture was maintained. Ainley-Walker [477] grew typhoid organisms in typhoid immune serum and concluded that this procedure effected a diminution in agglutinability, a heightening of its virulence and an increase in its resistance to serum protection. Bordet [56] pointed out that the inoculation of Metchnikoff's vibrio into animals already immunized caused a change in the organism in favor of greater virulence.

From these last mentioned results one may gain the view that there exists little unity in the reports on the influence of immune serum on

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* To the above it may be added that Julianelle [505, 506] has very recently shown that growth of the S type (capsulated) Friedländer's bacillus for 6 or 10 passages in broth containing 10% serum immune to the S type served to produce colonies of the R type (noncapsulated) culture. While the S culture was fully virulent, the R culture was without virulence.

the "normal" bacteral type, especially when the text books commonly intimate that the chief result of growing bacteria in immune serum is to increase their virulence. If one reviews these various reports with some care, however, it appears that these discrepancies may be explained by the following circumstance, which is shown with special clearness in the work of Steinhardt. Whether the normal S type will develop in its homologous serum and become more virulent, or whether it will develop in the serum and become less virulent (the latter reaction being accompanied by the S → R transformation) will depend on three circumstances: the actual titer of the immune serum, the concentration of the immune serum, and the length of time that the microbes are in contact with the immune serum (which must always be sufficiently fresh to permit continuous growth, preferably by means of successive passages). If the titer of the serum is too low, modification of the bacteria does not occur, except that they may become more virulent. If the serum is employed in too great a dilution modification does not occur (Park and Williams, Morishima), or perhaps the culture may become more virulent. If the time of contact of the bacteria with the immune serum is not sufficient, or if the number of passages through the immune serum (serum broth), is not sufficient, modification will not occur. This last result is best exemplified by the work of Metchnikoff [346, 347] and of Sanarelli [422] which led them erroneously to conclude that immune serum had no influence in diminishing the virulence of their treated cultures, but in some cases actually served to increase it. It is this old notion that has found its way into modern conceptions regarding the influence of immune serum on bacteria, as expressed by Zinsser in his textbook, by Stryker in her work on the pneumococcus and by many others. It has, moreover, doubtless been maintained in part by the somewhat natural expectation that there should exist a correlation between (immune) "serumfastness" and virulence in pathogenic organisms. That such a situation commonly exists for the bacteria at least seems very far from the truth, as shown by a great mass of evidence when it is correctly interpreted; although in the case of the pathogenic protozoa and particularly in the trypanosomes the situation is not so clear. Even in the action of immune serum against the cholera vibrio (the classical instance of bacteriolysis), although the vibrios disappear, it is by no means so certain that the culture is destroyed. We must come to the conclusion that there is considerable difference between the apparent lysis of living cells and their actual death; for, in such instances, there may be involved merely a transformation into another living form with

which we are only slightly acquainted. I suspect that this circumstance may have an important bearing upon certain problems in serum therapy.

We may thus conclude that, among the bacteria at least, wherever the question has been adequately studied and the results of the experiments adequately analyzed, the influence of a strong, homologous immune serum in continued application is such as to incite microbic dissociation, and thus to modify the virulent culture type in the direction of the R form; furthermore, that this resulting modification involves, in its more extreme aspects, far-reaching cultural changes, antigenic and serologic alterations of great significance and, finally, a loss of virulence which in some instances may be complete.

Pleuritic Fluid (in Tuberculosis).—Although we at present have little knowledge regarding dissociative reactions in the tubercle bacillus, it cannot be doubted that here, as in other types of infection, the eventual discovery of different forms of culture will sometime play an important part in problems of prophylaxis and therapeutics. So far as definite observations on the cultural behavior of B. tuberculosis itself are concerned, the chief support for this view lies at present in highly significant findings recently reported by Karwacki.[508] These concern peculiar cell reactions observed to occur in the tubercle bacillus when in contact, either in vitro or in vivo, with the pleuritic fluid of certain tubercular patients. Karwacki studied the reactions of 22 strains of the tubercle bacillus in 20 samples of pleuritic fluid drawn from tubercular patients. In 16 of these fluids the organisms underwent a "mutation" revealing giant forms, acidfast streptococcus forms and small cocci which might be either acidfast or cyanophilic. In many instances the transformation was accompanied by complete dissolution or lysis, and the author wonders if the now commonly observed filtrable forms of the tubercle bacillus may not have their origin in this reaction. In any case, the cultures often became "sterile" in the old morphologic sense.

The author also points out the very significant fact that there existed a distinct relation between the clinical character of the pleurisy and the transforming power of the pleuritic fluid obtained from the same case. The "mutation" reaction was observed most often in the fluid from the rapidly-terminating cases while, in the severe and chronic cases the action of the fluid was slight or absent. In such instances, however, the transforming action increased gradually and in the stage of resorption gave distinct transforming power. In view of these results

Karwacki believes that the transformation reaction possesses prognostic value, since the retrogression of the pathologic state depends in some measure upon "mutation" of the tubercle organisms into a nonacidfast form in which they are more readily disintegrated or destroyed.

While admitting the unknown nature of the transformatory and often bacteriolytic reaction, the author suspects that it may play a part in immunity to tuberculosis. Calmette has expressed the view that the lysins are incapable of modifying either the morphology or the physical properties of the tubercule bacillus; but with this view the findings of Karwacki are not in agreement, since they demonstrate the existence in the pleuritic fluid of a distinctly bacteriolytic agent, capable of accomplishing marked transformations in the type, and perhaps in the physiological reactions, of the tubercle bacillus in vivo as well as in vitro.

To the foregoing exposition it scarcely needs to be added that the important observations made by Karwacki are in harmony with much other work indicating the dissociation-furthering power of immune blood and of the body fluids of infected or partly immunized animals. If later study confirms the fact that Karwacki was dealing with the actual dissociation of the tubercle bacillus, as now seems most probable, and that this reaction involved distinctly protective aspects, we have here one more piece of evidence that at least one important defensive mechanism of the body against infection is the precipitation of microbic dissociation in vivo. In this connection I believe that Karwacki has opened the door to a new and significant field of investigation in the study of tubercular infections.

Products of Growth or of Dissociation.—It has long been recognized by bacteriologists that metabolic products might exert a strong repressive influence on the further development of the same, or on the growth of another, bacterial species. This subject has received interesting treatment by Eijkmann,[146] Rahn,[401] Hajós[229] and others, to say nothing of the more unusual features concerned with the filtrates of B. anthracis and B. pyocyaneus as studied by Emmerich and Löw,[158] Gamaleia,[194] Malfitano[315] and others. The effect of these growth products in modifying the form of culture has received less attention although it is undoubtedly of primary significance in many of the peculiar effects observed when different cultures are grown together in association, as in certain instances to be mentioned shortly. Indeed, our present knowledge of symbiosis and its effects in the case of pathogenic bacteria leaves much to be desired. It is the purpose of the present section, however, merely to indicate some of the examples sug-

gesting the influence of products of growth in promoting dissociations of the typical sort in sensitive cultures of several common bacterial species.

Quite recently Ørskov and Larsen[513] reported a peculiar instance definitely related to dissociation in a member of the paradysentery group, in which the filtrates of growth coming from a colony of a certain type ("B") showed definite inhibiting action on growth of the original culture. The subject is of such significance as to merit reporting in some detail. Plating a 14 day extract-agar stab culture gave two types of colonies, "V" and "B." The organisms in V were short and plump; those from B long and polymorphic—and often granulated or "degenerated," even in 12 hour colonies. The early V colonies remained normal for several days, but within this time the B colonies showed a "central decay" which later became macroscopically observable in the form of "fairly large holes." This lysis also occurred in V, but appeared later and was less pronounced. Seeding from these colonies gave colonies duplicating the original types. When picked to broth the V growth was homogeneous; the B growth spontaneously agglutinative.

After some daily streaks on agar each of these culture types gave rise to a new form, "M" and "Bu," respectively. On cultivation in broth V always split off M, while in agar stab it split off B. M and Bu, on the other hand, never gave any indication of returning to V and B, respectively. If V disappeared it was said to be impossible to obtain it again. The serologic reactions with these cultures are presented on an earlier page of this paper; also fermentation tests. The autolysis of the B type suggested the bacteriophage reaction and was therefore further studied. A 24 hour broth culture of B was filtered and a filtrate obtained which had a lytic effect most pronounced for culture M but also effective for a Shiga culture. "A few drops added to 10 cc. of bouillon simultaneously with the sowing of the bacteria in question completely prevented growth." A smaller concentration only delayed growth. No increase in the lytic agent was observed. These filtrates also exerted a restraining influence for growth on agar surfaces, one drop of a 1:200 dilution being able to accomplish this. After a few days scattered colonies often appeared on the lysed area, and these organisms were insensible to the same filtrate. Neither V, M, nor Bu formed such a lytic substance.

An interesting question arises as to the possible relation between the lytic substance observed by Ørskov and Larsen and the "alkaline-producing aërophilic ferment" studied in 1925 by Chiari and Loeffler.[98]

Here we observe a bacterial product of alkaline nature with which B. coli and other bacteria could be "infected" and made to perpetuate the phenomenon as revealed by whitish areas on Endo plates. Unfortunately we have no data regarding its effect on the morphologic, cultural or biochemical characteristics of the sensitive strain. Finally it may be added that de Kruif [120] found no stimulus to dissociation was given by growing his type D culture of B. lepisepticum in filtrates of type D culture. Enderlein [160] believed that metabolic products were able to incite the organisms of a culture to enter what he terms the "second reproductive stage," or the "Mochlolyse." From my understanding of this author, I believe that this is equivalent to what we have regarded as the transition from the S to the O or intermediate type. Enderlein's depiction of his various types rests purely on a morphological basis and thus renders some of his transitions difficult to follow. His culture types require correlation with certain biochemical and serologic characteristics before their relation to what we have termed the S, O and R forms can be entirely clear.

With further reference to the effect of metabolic products in producing dissociative reactions, there may be mentioned the recent report of L. Rosenthal [514] on B. anthracis. This investigator was able to change sporeforming races of this species to non-sporeforming races as a result of growing the cultures in the filtrates of previous cultures. Cultures were employed which gave abundant spores in about 24 hours under normal conditions. Such cultures were planted in the sterile filtrates of older cultures (either sporogenic or asporogenic) that had grown for about two weeks before filtration. In this filtrate the culture was grown for five days, then transplanted to a tube containing fresh filtrate. After three such passages the culture was plated and the colonies of the non-sporeforming bacilli selected. These, Rosenthal notes, can be detected after a little experience by the macroscopical features; but he fails to state what these differentiating characteristics are. If, however, his results conformed with those of Preisz, [393] Eisenberg [149, 151] and others, we may assume that the nonsporogenous colonies were of the translucent or bluish variety. The asporogenic nature of these cultures was demonstrated not only microscopically but also by the heating test. According to the view of the author, this new character was permanently acquired. In this case we manifestly have an aspect of the dissociative reaction in the bacillus of anthrax.

One of the most interesting cases which may appropriately be considered under the present heading involves the phenomenon of

"Entrainement" described by Et. Burnet [86] in 1925. As reported on a previous page (section 9) this investigator had already shown [85] that there commonly exist two serologic types of the organism of Malta fever, the "normal" and the "para" (Melitensis II) forms; and I have suggested already that the para type possesses many of the characteristics of the R form of culture, especially as described by Bassett-Smith.[39] Or at least it resembles a type of culture which shows the beginning of a transformation toward the R form. Burnet had already pointed out that one of the chief differential characters between these two forms was thermo-agglutination; that the para type gave this reaction while the normal melitensis did not. Indeed, the para types always seem to floc more readily than the normal. Heating was said to render the para form more agglutinable while it made the normal form less agglutinable.

The phenomenon of "Entrainement" appears to involve the more or less permanent modification of certain characteristics (biochemical, antigenic and serologic) of a culture through direct association with other cultures; or through submission (in the early stages of growth) of a culture to the filtered products of another culture of the same or a different species. In Burnet's elaboration of this phenomenon the first point of interest lies in the following circumstance. When normal cultures of melitensis were cultivated in association with the para form, the normal cultures became thermo-agglutinable—a characteristic of the para type. The experimental conditions necessary for performing this test of the results of the microbic association were realized in various ways. For instance, a collodion sac containing the normal melitensis culture was suspended in a tube containing a broth culture of the para form. After three to five days the normal culture was found to have acquired the property of thermo-agglutination. Similar results were obtained when Chamberland candles (porcelain) were used in place of the collodion sacs.

The first results were obtained by the use of living para culture, but Burnet was able to demonstrate that it was not necessary that the para culture should be living in order to produce these results, for cultures killed by heating at 65 C. accomplished the same effect. After three or four days the normal melitensis was found to manifest thermo-agglutination. It is of special interest that Burnet states that it is necessary in all cases that there should occur a "development" or growth of the normal culture in order that the modification may be accomplished. A few minutes contact was not found to suffice. Here perhaps we may

recall that it is always necessary for cultures to grow before they can dissociate; and that it is equally necessary for them to grow before they can undergo transmissible autolysis.

Another point of interest as described by Burnet is the following. The phenomenon of "Entrainement" may not only be "specific" (para-melitensis vs. normal melitensis), but may also be "non-specific" (B. anthracis or B. pestis vs. normal melitensis). One suspends a normal culture of melitensis, already partly developed (in a collodion sac), in the filtrate of a culture (or in the broth culture itself) of B. anthracis or B. pestis, and allows the development of the melitensis culture to proceed for some days. At the end of this time the culture is tested and is found to give a positive thermo-agglutination reaction, while it did not give this reaction at the beginning of the test. The normal culture had thus been transformed into a culture possessing one of the distinguishing features of the para type.

Regarding the permanence of the newly acquired and distinctly "heritable" character, Burnet stated that, when the thermo-agglutinating characteristic had once appeared in a culture it could be transmitted indefinitely through subcultures. The modified melitensis ("entrainés") cultures were still thermo-agglutinable twenty generations away from the original, normal stock. The stimulus was always found to operate from the para form to the normal culture; never in the reverse direction.

Although "Entrainement" was found to concern in all cases the phenomenon of thermo-agglutination, it was not related exclusively to such physical properties of the culture, but to the biochemical and antigenic characters as well. With thermo-agglutinability were found to be correlated other characteristics, such as specificity in sero-agglutination, feeble production of agglutinins in rabbits, agglutination in normal sera and a tendency toward spontaneous agglutination. The melitensis culture modified ("entrainé") by association with either the para form or with cultures of other bacterial species, such as B. anthracis, B. pestis, V. cholerae or B. typhosus (also possessing the property of thermo-agglutination), was found to have gained also these other characteristics. With reference to the serologic tests, it was observed that the modified melitensis culture no longer agglutinated in rabbit serum immune to the normal melitensis culture. At the same time, such a modified culture was reported not to have been made more agglutinable with rabbit serums immune to the para culture type itself. In the light of other dissociative reactions, this result is somewhat peculiar.

Antigenic modifications of normal melitensis were also secured through association with cultures (alive, or killed by heating), or with culture filtrates of other bacterial species (B. anthracis, B. pestis, etc.). The modifications produced in this way, however, were less constant and less pronounced than those produced by association with the paramelitensis culture. When "Entraînement" was effected with killed cultures of other species, it often required several passages in series of the normal melitensis culture (in sacs or in candles) through the heterologous culture, or culture filtrate, before an appreciable antigenic modification in the normal type was obtained. Here we may bear in mind that, in the common dissociative reaction, the transformation from the S to the R form of culture is not attained at once but ordinarily by slow stages.

In his discussion of these results, and of the nature of the phenomenon which he describes, Burnet undertakes to relate them to other reported cultural transformations cited in the literature, but does not approach our present views regarding microbic dissociation, and indeed does not see his way clear to any satisfactory explanation. It seems to me, however, that just as the transformation of the normal melitensis culture type to the para form involves in all probability the essence of the dissociative reaction, so also in all his cited cases of modification through culture association, whether with the same species (para type) or with other species (anthrax, pestis, cholera, typhosus), we are confronted only with a dissociation of the normal, or S type, of melitensis into the R state, or into a form approaching the R. We have seen in this section that microbic dissociation is a phenomenon that can be "forced" by many different agents, chemical, physical and biological. That certain metabolic products given off from R type cultures are able to repress and to modify the growth of S culture of the same species has been pointed out by several investigators. Through the work of Burnet it now seems assured that another highly important "reagent" capable of instigating the dissociative reaction may be present in the growth products or dissociation products of heterologous bacterial species. The banal influence of pyocyaneus and anthrax filtrates on alien cultures is now well known through the work of Emmerich and Löw [158] (pyocyaneus) and of Malfitano [315] (anthrax) ; but in these cases it has never been demonstrated that the pyocyaneus and anthrax filtrates produced what we may now term cultural dissociations, although this seems probable. It is thus to the credit of Burnet's highly interesting study that we are now furnished with clear evidence of the importance of

alien culture filtrates in initiating, in sensitive cultures, many of the reactions characterizing the phenomenon of microbic dissociation.

Although Burnet no doubt has presented one of the clearest instances of dissociation resulting from the use of heterologous cultures and culture filtrates, there exist in the literature other instances in which the same mechanism may be in operation, producing effects which are apparently unusual and difficult to explain. For example, Schiller [432] in 1914 studied the influence of cultures of B. acidophilus on the growth and viability of various streptococci. He observed that, if any streptococcus culture was added to a culture of B. acidophilus, the streptococci were killed in 18 hours at 37 C.; and apparently by lysis. After a week the streptococci were reduced to an amorphous mass. Schiller could not obtain similar lytic action in any other way. The fact that the results were not attributable to the acid elaborated by acidophilus, as one might expect, was shown by the circumstance that the streptococci would grow in acidophilus filtrates. But they would not grow in a filtrate of a broth culture of acidophilus in which streptococci had previously undergone autolysis. Thus: to an 18 hour broth culture of acidophilus was added a mass of streptococcus. This was destroyed in 36 hours. From this culture was then obtained a filtrate which proved just as germicidal to the streptococcus as the mixed culture of acidophilus and streptococcus. Schiller thought that the streptococcus might provoke the secretion of a defensive bactericidal substance on the part of B. acidophilus. That the phenomenon was not due to disintegration products of streptococcus itself was clearly shown by growing the latter in heated and aged streptococcus filtrates.

Also Castellani [91] in 1925 described some curious results observed in bacterial cultures grown in association. His work appeared to demonstrate that when two bacterial species grow in association, the resulting culture form comes to present biochemical characteristics which are possessed by neither of the original cultures when grown separately. B. typhosus, for example, grown under ordinary conditions, gives acid but no gas from maltose. The Morgan bacillus gives on maltose neither acid nor gas. But, when these cultures were grown in association in a peptone-maltose medium, the combination produced both acid and gas. If, however, the Morgan bacillus was added 24 hours after B. typhosus no gas was observed. In another case B. dysenteriae Flexner and the Morgan bacillus were grown in association. The former gives acid but not gas in mannitol. The latter gives neither. But, when the two grew in association, the combined culture yielded both acid and gas from the

same medium.   Phenomena of this sort are admittedly difficult to
explain.   Without the necessity, however, of calling to our assistance the
"hybridization theory" of Almquist,[6] and in view of the modifying
effect, biochemically and serologically, of the dissociative process (in
which we know that fermentative reactions may undergo radical
changes), it might perhaps be shown that the new culture form present-
ing the new feature of gas production was merely a dissociate (R or
other) from one of the principle organisms employed in the tests.   The
exact nature of these phenomena is still obscure, but opens a field of
study possessing much interest.   The same may be said of Gratia's work
showing the antagonistic and even lysogenic action of certain molds on
heterologous species of bacteria.

Another interesting instance of culture modification through associ-
ation is one recently reported by Jeanne Lommel.[511]   Tubes of broth
were inoculated with B. typhosus, B. paratyphosus, or B. dysenteriae
Shiga.   After the growth had attained about 500 million bacteria per
cc. the tubes were inoculated with B. coli.   From this point on, the
method employed does not receive a clear presentation, but the state-
ment is made that the B. coli used became modified to the extent of
being able to ferment sucrose, which the original culture did not attack.
The coli strain was therefore transformed into B. coli communior.
Similar results were obtained by continued growth in broth contain-
ing phenol; also in broth containing formol.   Since this sort of microbic
association must occur in the intestinal tract whenever an infection with
B. typhosus, B. paratyphosus or B. dysenteriae Shiga occurs, the author
concludes that B. communior is merely a "simple biochemical deviation"
from normal B. coli.   In view of the results presented by Et. Bur-
net,[85, 86] it would be of interest to ascertain whether the transformation
observed by Lommel might not also be effected by filtrates of the cultures
employed, as well as by the whole cultures.

Taking these results in conjunction with those of Esther Stearn,[452]
we can now conclude that B. communior can be produced from B. coli in
three ways: through the action of gentian violet; through the action of
phenol or formol; and through the action of microbic associations.

In concluding our consideration of the effects of microbic associ-
ations a recent contribution by Holman and Meekison [504] should be men-
tioned.   These writers have brought together certain of the earlier
observed facts relating to the effects of microbic association, together
with some observations of their own.   They point out anew that two
bacterial species living in association may bring about biochemical

changes which neither is able to produce alone. To this phenomenon the writers give the name, "synergism." Under this heading they list among others the observations of Castellani mentioned above. Prominent among the personal instances cited by them is the production of gas by the association of B. coli and S. fecalis in a medium containing saccharose. In this connection they state that the gas production was checked by increased acidity but that the addition of alkali would start the process again.

In attempting to explain these reactions Holman and Meekison, although making allowance for "changes in the metabolism of the bacteria" (implying, as I understand it, a change in the inherent physiologic reaction), seem more inclined to regard the mechanism as one involving a "co-operative" action on certain constituents of the medium. They state: "One of the pair of bacteria must be capable of splitting the test substance and forming acid. The other must be able to form gas from monosaccharides." In other words, one of the pair must be able to execute some sort of a preparing action for the work of the other.

In many of the numerous symbioses reported as existing between microorganisms of diverse nature, and also in some of the instances cited by Holman and Meekison, there seems little doubt that such an influence takes place. Moreover, that it is manifested not only by the production of gas from a certain sugar by the associated pair when neither of them alone produces gas, and only one of them produces acid, but also by the production of acid from a certain sugar by the associated pair when neither produces acid alone (Jeanne Lommel [510, 511]). The sort of reaction described by Holman and Meekison may occur in the majority of such associations; and for this phenomenon "synergism" is certainly an appropriate name. On the other hand, our present knowledge of dissociative phenomena may prompt the inquiry whether all such apparently synergistic phenomena possess the same significance. May not another reaction appertaining more closely to microbic dissociation be concerned in some of these instances? Reasons for doubting the unity of the phenomenon in question as described by the above authors may be found in the following circumstances. We know that B. coli is capable of transformation into a saccharose-fermenting (communior) type. We know, moreover, that the agencies by which this modification may be accomplished are diverse. It may be accomplished by continued growth in a medium containing malachite green (Jeanne Lommel [511]), by long submission to the influence of gentian violet (Esther Stearn [452]), by the continued influence of phenol or of formol (Jeanne Lommel [510]).

The last mentioned investigator showed further that the modifications
produced by phenol and formol were less permanent than those deter-
mined by the influence of microbic association of B. coli with B.
typhosus, B. paratyphosus A and B, and B. dysenteriae (Shiga) after
twenty or more passages. In Lommel's case it is true that the study did
not involve fermentation of saccharose with gas production; but the
essential fact is that a new fermentation power was manifested by the
associated pair, or by the single organism (B. coli) under the influence
of an unusual environment.

It thus appears that a transformation of B. coli with respect to its
fermentation abilities can be determined, not only through microbic
association, but also through the presence of various antiseptic sub-
stances in the medium in which it is grown through a series of gener-
ations. In the latter cases there is manifestly no opportunity for
"cooperative" bacterial action. Here we must assume either that the
added reagent (dye, phenol, formol, etc.) serves to generate in the
medium a new fermentable substance, fermentable by the unmodified
culture; or that the organism concerned becomes modified insofar as it
gains the capacity to ferment the original, unmodified substance. In the
instances reported by Stearn and by Lommel, and in some of those cited
by Holman and Meekison, it seems to me more likely that it is the
latter reaction that really obtains. And added evidence favoring this
view lies in the circumstance that such transformed cultures are likely
to vary, not in one but in several respects, from the original type—
undergoing a loss of some characters and a gain of others. If such
transformations can result from the addition of certain chemical sub-
stances to the medium, it is not too much to expect that they may also
occur under the stimulus of microbic associations.

In concluding this aspect of the matter I may say that the reactions
included under the head of Holman's and Meekison's "synergism" are
doubtless highly intricate, and that it may be desirable, pending the
acquisition of further data, to hold open the possibility that phenomena
of this apparent sort may embrace a variety of causes. Modification of the
culture type through microbic dissociation (with consequent alterations
in inherent fermentative capacity) determined by microbic associ-
ations may occur more frequently than Holman's and Meekison's inter-
pretation of the reactions would permit us to be believe. Such reactions,
involving dissociations, could therefore not properly be regarded as
synergistic in the sense in which this term has been introduced. It
seems to me that the question of synergism thus resolves itself into the

true and the false. The true synergism would be that in which, as Holman and Meekison have clearly indicated, there exists a "cooperative" reaction but without necessary modification in either of the associated bacterial types. The false synergism would be that in which the outstanding results are referable to a modification of one or both of the associated pair through the functioning of the dissociative mechanism. The two sorts of reaction are manifestly quite different, and clearly to distinguish between them will be the necessary task of future workers in this interesting field of investigation.

The Influence of Certain Colony Types on the Culture Substratum.
—The consideration of secondary or daughter colonies presented in earlier pages of this work serves to demonstrate that there may reside within the substrata of apparently homogeneous culture certain localized centers where a small group of organisms of a quite different type and potentiality from that of the mother culture have become differentiated and are carrying on their activities. If they remain few in number, and are not differentiated by color differences, as in the case of B. prodigiosus and other chromogens, they may escape unobserved. If they can successfully oppose the surrounding culture and increase to a sufficient extent, they produce a visible colony lying imbedded in the larger culture mass, which may be represented either by the mother colony or by an extended growth on agar surface. They may even appear as easily differentiated colonies of an agglutinative type in a liquid medium. What effect these secondary colonies exert on the primary growth might be expected to depend on their biochemical constitution, and on the kind and degree of the opposing biologic factors concerned in the association. And the same might be said with reference to the effect of the primary colony (or primary culture growth) on the secondary. It does not require a great stretch of imagination to suppose that, depending on the balance of those biologic factors underlying the lysogenic function, either the secondaries might destroy the primary culture, or that the primary culture might destroy the secondary, although this is less probable. As a matter of fact, among the great number of instances in which secondary colonies have been observed, and in a considerable number of bacterial species, neither of the reactions mentioned above is known commonly to occur. It can be stated with assurance, only that when colonies of the R type arise in a primary growth comprising the S culture, the R secondaries possess much greater vitality and still survive long after the S culture has perished. This was indicated strikingly by the studies of Atkin [22,21] on the gonococcus and

the meningococcus.   When, however, S type or O type cultures arise
as secondary colonies in R substratum, we are not so sure of the sequel,
because the exact conditions underlying such instances have not been
clearly recognized or studied.   Thus, although little can be said defi-
nitely on these matters, there are a few cases in which it is strongly
suggested that certain repressive, and perhaps lysogenic, influences of
the sort mentioned above actually exist.   For this reason our consider-
ation of the incitants to microbic dissociation would not be complete
without reference to certain observations dealing with the influence of
some slightly known types of colony, resulting from dissociation, upon
the other S members of the bacterial society.   This aspect is obviously
closely connected with the matter just discussed, and is, I believe, likely
to play an important rôle in further study of transmissible autolysis.

The problem in its present state is difficult to present because it often
is impossible to draw sharp lines between the rôle of metabolic products
and more specific inter-colonial influences.   The essence of the problem
was first made clear by the studies on B. anthracis by Preisz,[393] Pesch,
Katzu and others, dealing with the lytic spots arising in cultures on
agar.   De Kruif,[120] however, was one of the first to call attention to a
related phenomenon in cultures in which the chief dissociates were
clearly recognized.   When a culture of Bact. lepisepticum containing a
mixture of S and R, but with a preponderance of the latter, was streaked
parallel on an agar plate, both forms developed their characteristic
colonies.   But the interesting point is this: The first two or three
streaks on the plate showed massed R colonies, but no S, although S
colonies were numerous in the subsequent streaks.   If S colonies did
appear in close contact with R colonies the former were irregular and
distorted, their margins being cut into by the R forms.   Such pictures
give the impression that the latter exert a strong repressive influence
on the development of the S.   I have seen the same phenomenon
repeatedly in mixtures of R and S of many bacterial species, including
intestinal forms, B. diphtheriae, B. proteus and B. subtilis.   Mr. Nun-
gester in our laboratories has made a similar observation, to be reported
later, on dissociating cultures of B. anthracis.   Pesch [387] stated that the
greater development of the "blue" or transparent (R) form of this
organism could "repress" the development of the smooth, opaque form
(S).   Mellon also has mentioned the antagonistic action of certain cell
types upon the growth of other forms of the organism in the same cul-
ture.   The matter has also been briefly referred to by Ischii [262] for B.
typhosus.   Gildermeister,[199] moreover, described for B. coli a special

type of colony which he termed the "insonstant form" or "Flattenform" which changed rapidly in subculture and which possessed lysogenic tendencies. D'Herelle [248] believed this culture was "contaminated" with the bacteriophage.

Arkwright,[17] furthermore, has added evidence of the repressive rôle of certain colony types in Shiga cultures. He mentions certain "small colonies which appear to have an especially inhibitory or lytic action on larger colonies present on the same agar surface. The small colonies lie in concave notches of the larger ones." Such variants, he pointed out, like sensitive variants, may arise either from normal cultures or from cultures submitted to the action of the lytic principle. "The behavior of these variants indicates the action in the cultures of an excess of bacteriolytic function in some or in all of the bacteria present. . . ." It may be added that Mr. Nungester in this laboratory has demonstrated in his study of anthrax dissociation a third type of anthrax colony, sometimes very numerous on plates, and characterized by self-lysis. In further course it becomes transformed into the R type culture as well as continuing to perpetuate the lytic type. The lysogenic function of these colonies has not yet been studied.

Perhaps somewhat related to the antagonistic influence of certain colony types upon the mass of S type culture are the observations of Gratia [213] on the antagonistic influence of one culture of B. coli upon another coli culture. Strain V grew normally and when implanted in broth gave normal clouding after three hours. When one drop of the filtrate of V was added to a broth tube freshly implanted with another coli strain (ϕ), no growth appeared for seven hours and then a spontaneously agglutinative growth appeared. When a drop of the filtrate V was allowed to run over an agar slant recently inoculated with strain ϕ no growth appeared on the drop area. Finally the clear area yielded a few colonies which, when seeded to broth, gave an agglutinative growth. The V principle was not generated by strain ϕ, and that generated by V showed no possibility of serial transmission. Whatever may have been the cause of the reaction in strain ϕ culture, Gratia's results indicate a transformation into an R type of growth; in other words the bringing into effect of a marked dissociation under the influence of V or its filtrates. It is regrettable that so interesting a phenomenon was apparently not further studied.

Conclusion.—In concluding this section on the incitants to dissociation I wish to add that I have used the terms "to incite" and "to enforce" dissociation as if the action of the incitant were direct in deter-

mining the generation of the transitional or the R type culture. It is doubtful, however, if any such direct action occurs. It seems much more probable that the critical act of dissociation, involving what it may, is accomplished by the bacteria for themselves; and that the "inciting substance" or "inciting condition" merely sets into operation a physiologic mechanism by whose activity or by whose products the ultimate results are accomplished. Moreover, it seems that when this mechanism is once set into operation it tends to maintain its action until a certain portion of the culture is exhausted and certain transformations accomplished. My meaning in this will perhaps become more clear as we advance to later sections of this paper.

The problem of the action of incitants is undoubtedly capable of solution; but that the means and the results may be found different for each unrelated group of cultures we can scarcely doubt. We can even now observe, however, that one common factor unites all the agencies that we have thus far surveyed—namely, a condition unfavorable for growth of the S type culture. As a consequence it passes into a transitional form which is highly unstable; and from whose destruction in greater or less degree there arises a new type, the R. When the nature of this reaction is discovered it may be anticipated that the path will be revealed to the solution of another phase of the problem of dissociation —namely, the phenomenon of the bacteriophage.

Of the data that have been presented, probably the most significant relate to the action of immune serum on the virulent S type of culture. We have seen that the common effect is to produce a culture of modified antigenic nature, of diminished virulence and of increased phagocytability; moreover, that there is some evidence to suggest that this modification occurs in vivo as well as in vitro. These results are therefore naturally concerned with a very fundamental aspect of immunity— namely, the rôle of the immune serum and its actual mechanism in protection. Out of the vast amount of study which this last problem has received during the past forty years, a lamentably small nugget of truth has been derived. We have been forced to abandon many of the older conceptions regarding the bactericidal influence of immune serum so eagerly supported by numerous older writers; but little has been supplied of a direct nature to take their place. The accent given to various theories of immunity has changed with the season, although in more recent time there has appeared a definite tendency to turn more and more to the influence of the bacteriotropic antibodies in their correlation with phagocytosis. But the bacteriotropic antibodies are not ordi-

narily germicidal, although they are sometimes bacteriolytic. They are usually conceded to be agents which effect some sort of a modification of the virulent culture preparatory to phagocytosis, but not necessarily destroying directly the virulent organisms. What is the nature of this "preparing action" on virulent bacteria? I believe there already exists sufficient evidence to intimate that it is *incipient microbic dissociation in vivo;* and that this reaction is the underlying mechanism of all opsonic action. This possibility was first suggested by Griffith [215] in 1923.

If this view of the matter should eventually prove to be true, it is possible that the aim of much therapeutic endeavor might undergo significant modification. At the present time we often seek to destroy microorganisms which have established an infection in the blood or tissues and we have become accustomed to measure the expected efficiency of the destructive agents by ascertaining their germicidal power in vitro; we look for the killing power of the substance and trust that the effect in the body may be on the same order. It might be the outcome of such a new conception as I have outlined above, that we should strive not so much to destroy directly the microbes infecting the blood or tissues, as to stimulate dissociation in vivo; and so permit the organisms to work out their own destruction while aided by the phagocytes.

Although we know little at present regarding the influence of various substances, organic or inorganic, in effecting microbic dissociation in vitro, and even less regarding inciting the reaction in vivo, it might eventually appear that many of the substances that are at present employed because they are germicidal or bacteriostatic in vitro will be found to have less significance as stimuli to dissociation than some others that may have no or slight germicidal power. One reason for considering such a possibility is that, while the average homologous immune serum cannot be said to possess appreciable germicidal power, we know of no incitant to dissociative reaction, either in the tube or in the body, which can compare with homologous immune serum in energy, speed of action or selectivity when it is employed in sufficient concentration and for a sufficiently prolonged period.

These conceptions of the possible part played by microbic dissociation in vivo as a protective reaction, and of the homologous immune serum as a dissociation-provoking agent, are as yet far from possessing adequate support in concise experimental evidence. But it may be said with some justification, I believe, that the view is sufficiently well grounded to warrant its consideration as a modifying factor in many aspects of experimental immunization and serum therapy.

On the other hand, there is one circumstance that may cause us to be less optimistic regarding the possibility of creating therapeutic dissociations in the body except in a few infections in which we can recognize the S type culture as most virulent. Experience leads us to believe that several pathogenic organisms whose S type carries the virulence are taken into the body in this form, and in such case may eventually be dissociated into the nonvirulent and easily phagocytable R form under the influence of the blood, immune bodies or body fluids. But suppose, as might easily be the case, we are dealing with pathogenic organisms in which the virulent stage in the cyclogeny of the species does not lie within the S type culture, which in this event may be nonvirulent. It is then conceivable that such organisms, taken into the body in a harmless state, might, under the influence of the blood or body fluids, be dissociated into a virulent form. From this point of view the socalled filtrable virus might represent an ultramicroscopic and virulent dissociate of an organism which is common to us in the guise of a microscopic and nonvirulent form. It seems more than possible that these views may have a relation to several types of streptococcal and of spirochaetal infection. This conception is admittedly highly speculative, but it has some basis in fact. Furthermore, I believe the knowledge already gained of many remarkable phenomena occurring under the cloak of microbic dissociation should make us alert to eventualities heretofore unsuspected in the field of pathogenic bacteriology; and particularly with reference to the filtrable forms of pathogenic bacteria.

12. THE DEGREE OF PERMANENCE IN THE CHARACTERISTICS OF THE R DISSOCIATES AND THE NATURE OF THE R → S REVERSION

With reference to problems in epidemiology and carrier infection microbic dissociation assumes considerable significance; and in this relation one of the most important aspects lies in the question of the permanence of the characteristics of the R types and other less clearly recognized culture forms. Moreover, the question of permanence bears closely upon our current views of bacterial mutation, since the justification with which we might regard the newly-formed dissociates as true mutants, would depend upon their ability to hold permanently to their newly acquired form, and to become the center of fresh variational activity—a point considered in another section of this paper.

Evidence for Permanence of R Type.—Regarding the permanence of the R types there exists much conflicting evidence. Firtsch,[178] who in 1888 was one of the first to present evidence of the phenomenon that

we now term dissociation, spoke strongly in favor of the permanence of his types ("variants") II and III of the Vibrio proteus. Dyar,[142] who in 1895 presented the details of a clearcut case of dissociation (in this case involving B. lactis-erythrogenes), stated that, of 125 subcultures made from isolated colonies of the "wrinkled mutant" (R), all but three remained true to the wrinkled type; and these three were of the normal, "soft" form (S). At the same time Dyar stated that a few of the wrinkled colonies showed "soft borders." The wholly wrinkled type, however, bred true for many generations, and in the hands of a less careful observer than Dyar might easily have been reported as involving a permanent mutation. Dyar apparently recognized nothing unusual or significant in his findings and casually remarked that the same sort of phenomenon could probably be observed in most bacterial species. After more than thirty years, we are just beginning to appreciate the truth of this view.

Loeffler[307] also reported that his four B. coli mutants remained true to type. Baerthlein[30] recorded permanence for some bacterial species but not for others. The permanence of what was probably the R type of the diphtheria bacillus has been upheld by Corbett and Phillips,[110] Slawyk and Manicatide,[443] Zupnik,[498] Bernhardt[49] and many others; and most recently by Crowell[114] working with a single cell strain. Von Lingelsheim[304] reported the permanence of his "Q-form" (possibly an "intermediate") of B. typhosus for at least five years and Feiler[167] recorded the permanence of his immune serum R strain of the same organism on agar, though not in broth. R types produced by Feiler by other means (starvation, phenol) were not so well "fixed." Bernhardt stated that no reversion of his "extreme variant" of typhoid occurred in 15 months. Arkwright[16] mentioned the permanence of the R form of various members of the colon-typhoid-dysentery group in some cases but not in all. Schütze[436] stated with reference to B. paratyphosus B that "it is impossible apparently to convert a 'rough' into a 'smooth'". Preisz,[394,395] Eisenberg,[149] Bail and Flaumenhaft,[34] Wagner[476] and others found the nonvirulent type of B. anthracis (which as we have seen seems to correspond to the S form) remained constant after long isolation and after many transfers. Weil and Felix,[483] Braun and Schaeffer[66] and others bespeak a high degree of permanence for the O form of B. proteus X19 and X2. P. B. White[487] attempted by many methods to cause a reversion in his R strains of B. cholerae suis but without success and concluded: "On the whole we are of the opinion that the rough variation is probably an irreversible change." In the

same organism Orcutt observed a considerable degree of permanence. De Kruif [119] found no evidence of the return to the S form of his R type of Bact. lepiseptieum, except in a few cases of animal inoeulation which, however, (because of the frequency with which normal rabbits earry the S organism) do not constitute altogether favorable evidence. All cultivation methods were reported unsuccessful in causing reversion in this ease. Reimann, [405] as also Amoss, [8] have favored the view of the permanence of their R types of the pneumococcus, whieh Amoss regarded as "a genuine mutant." Mary Cowan [111] also considered her R type streptococci (both hemolytic and greening) as fixed stages. I may add that I have maintained for four years R strains of B. pyo-eyaneus without there having occurred any tendency to revert to the normal pyocyanogenic (S) form. An R type of the Friedländer bacillus (noncapsulated) in my hands has maintained its chief characteristies for more than two years.

Hans Wreschner [492] in 1921 presented evidenee dealing with the permanenee of the noneapsulated (R) type of M. tetragenus and its failure to revert to the eapsulated S type. He found that in blood mediums such as serum broth the reversion occurred regularly in most eases; but a strain of R was ultimately found which no longer possessed the ability to revert in any medium. Further data on Wresehner's interesting case are presented in eonnection with eapsules (section 5) and with virulence (section 8). Eisenberg [153] in 1914 also recorded the permanence of the R type of M. tetragenus but did not study the question fully.

To the views mentioned above relating to the degree of permanenee of the R type cultures arising from active microbic dissociation, a state-ment should be added regarding the permanence of the presumably analogous R type cultures arising under the influence of the baeterio-phage; for, as we shall see in a later seetion, the phenomenon of trans-missible autolysis, accompanied by its generation of secondary, resistant eultures, in reality may be regarded as a form of dissociation of the original sensitive culture. D'Herelle does not bring out this relation, and for the reason that, throughout all of his publieations, he is appar-ently oblivious to the existence among bacteria of the phenomenon of microbic dissociation which receives no mention, either under this cap-tion or under any other, in any of his works. For him, a resistant cul-ture is always one that has been made so through previous contaet with the bacteriophage. He [248] notes, however, that these secondary cultures are often quite different, morphologically, biochemically and serologic-

ally, from the original culture. They are all indeed "mutants," immune strains, produced by the bacteriophage! Among them some may revert easily and quickly to the former state while others he regards as permanent in their newly acquired characters. We shall see later, in the section dealing with the relation of dissociation to the mutation theories regarding bacteria, that Bordet [61] also has raised the bacteriophage to a high eminence as an agent producing permanent mutations, and thus controlling the destiny of the species. But, as we shall also note later, both of these views are alike in their falsity. True bacterial mutations are, as yet, unknown, for we have been using false criteria for their attempted recognition.

Evidence for "Reversion" of R Type.—Regarding the reversion of the R form to S, fewer instances are on record. Bordet and Sleeswyk,[64] it is true, observed the variant of their B. pertussis return to the original form as soon as it was again placed on blood medium (from plain agar); but Bordet and Sleeswyk did not work with pure line strains. Penfold [386] found his daughter-colony mutants (the typhoid isodulcite fermenters) reverted easily unless grown for a considerable time on the same medium that gave the mutation; and, even under these conditions, partial reversion was often observed. Baerthlein [30] who gave the subject of reversion considerable study reported the return of the R type of his B. paratyphosus B after five and one-half months of uninterrupted cultivation in broth. Wreschner [492] obtained the reversion of one R form of M. tetragenus, but not of another R form of the same strain, by cultivation in normal serum broth.

In the case of the pneumococcus the reports regarding reversion are variable. Notwithstanding the permanence of the modified type obtained by Stryker [454] by the employment of homologous immune serum, so long as the culture was maintained on plain mediums, she found that inoculation into mice determined a reversion after one or more passages. The number of passages required to accomplish this seemed to depend, however, on the length of time that the modified culture had been under the influence of the homologous immune serum. If the original virulent culture had received only 6 to 12 passages, one passage through a mouse might reestablish a certain degree of virulence. If, however, the culture had received 50 to 100 passages through immune serum broth, three or more passages through mice were required to bring about a reversion to the virulent form. Here we manifestly have different degrees of "fixity" of the variant which are quite in accord with the

results of much work of a similar nature on other microorganisms.
Unlike Griffith, Reimann and Amoss, to be mentioned presently,
Stryker did not ever observe absolutely irreversible pneumococcus vari-
ants. One possible reason for this difference is a circumstance pointed
out seven years later by Griffith; namely, that the constancy of the
R variant in its newly acquired characteristics is determined, among
other things, by the strength of the immune serum employed. Griffith
particularly was careful to impress upon his rabbits, by means of many
injections, the highest possible grade of immunization. That Stryker
took unusual precautions in this respect does not appear from her experi-
mental records.

Although both Reimann and Amoss reported the stability of their
pneumococcus R types, Griffith was more conservative and his results
are of special interest. He [215] pointed out that the permanence of the
R form depended in considerable measure upon the manner of its pro-
duction, agreeing in this matter with the view of Braun and Schaeffer
for proteus and with Feiler for B. typhosus. We may use his own
words: "A rough colony culture which has been produced from a
virulent strain by a single passage through immune serum, may retain
its rough character for many generations in plain blood-broth and
. . . may subsequently revert to the smooth type." Under these
conditions virulence was recovered. He stated that this result obtains
especially with the type 2 pneumococcus. But again he stated: "The
R type characters, after three passages through immune serum broth,
have remained constant for more than 13 subsequent generations in
plain blood broth. The height of the serum titer seems to have an
influence on the permanence of the change." One other interesting
point is added. After long cultivation in plain blood broth certain of
the R colony strains became in appearance almost indistinguishable from
the S strain, but remained attenuated. This observation, at present
unique in dissociation records, may give a hint as to the origin of
nonvirulent pneumococcus S types. So far as the features of the
special pneumococcus antigen involved in virulence are concerned,
Griffith believed that the most important aspect of the $S \rightarrow R$ trans-
formation was the loss of ability to produce the specific soluble sub-
stances. Evidence for this was also presented.

The study by Feiler [167] on reversion of B. typhosus is of special
interest. He obtained modified typhoid cultures by means of phenol agar,
"starvation agar" (see Braun and Schaeffer on proteus), and immune
serum. In the first case the dissociation apparently progressed only to

the intermediate O forms, while in the last the true R form was undoubtedly obtained. When Feiler attempted to obtain a reversion by returning the modified cultures to normal culture mediums, this was accomplished easily with the starvation strain, with greater difficulty in the phenol strain, and with still greater difficulty in the case of the immune serum strain. The last, after 107 passages in inactive, immune serum broth, returned to normal only after 18 passages through broth without serum at 37 C. At 22 C. twenty-six passages were required. When a reversion of the immune serum strain was attempted on agar, 25 passages failed to produce the normal type; the modification thus appeared permanent under these growth conditions. From these results we can see that growth on a solid medium may favor, not only the stability of the culture against the dissociation from S to R, but also stability against reversion from R to S. It is also of interest to note that, just as Soule [450] observed that the S → R transformation in B. subtilis occurred more rapidly in large volumes of medium (300 cc.), Feiler found the R → S transformation of B. typhosus was hastened by transfers through comparatively large volumes (50 cc.). Passage of the R typhoid through guinea-pigs showed no influence in effecting reversion.

One of the more recent instances of reversion of type R culture is found in the work of Jordan [273] who studied the problem in B. paratyphosus B. For this study he used two representative cultures which at the outset showed respectively 10 S to 1 R, and 5 S to 1 R, as indicated by examination of colonies after plating. From these cultures single cell isolations were made and pure cultures of S and R obtained. Frequent transfers were then made through veal infusion broth, P_H 7.4. One of the R strains revealed some S colonies after 16 days (32 transfers); the other after 25 days (50 transfers). The virulence of the derived S type (after it had been regained from the R) was still intact, while the accompanying R form was still nonpathogenic.

Most recently Soule [450] has accomplished the reversion of an R type of B. subtilis which had remained constant on solid media for many generations. The reversion was effected by the addition of 5% of active, normal rabbit serum to the beef infusion broth used for culture. After eight hours in such serum broth the degree of dissociation amounted to about 10% of the cells, as indicated by plating; while a control tube without serum, placed under the same conditions for growth, remained 100% R type cells. Stronger concentrations of normal serum were not found to produce a more marked reaction. When, how-

ever, a 10% homologous, R immune serum broth was employed, a
reversion of about 40% of the R cells was quickly obtained.   This
interesting result has been considered more fully in section 11.   It is a
matter of some interest that something in serum is able to enforce the
R → S transformation, not only in pathogenic species like the penumo-
coccus and lepisepticum, but also in so prominently a saprogenic form
as B. subtilis.

Conflicting Evidence.—A consideration of the nature of the incitants
to dissociation, and the possibility of reversion, would not be complete
without the presentation of certain evidence which in some measure
tends to confuse the fairly definite results already established.   The
observations I refer to are found in the work of de Kruif [120] and
Morishima.[352]   The point involved in both is the following: not only
was dissociation produced, but after a time, although the modified
culture was maintained constantly in the same sort of medium that
enforced the dissociation, a reversal to the normal form seemed to
occur.   This seems fairly clear in de Kruif's work; in Morishima's
observations another explanation is perhaps possible.   The instances
may be described as follows:

De Kruif studied the influence on the D → G (S → R) transformation of
varying amounts of peptone, 0.2 to 2%, and made readings of the degree of
dissociation, in terms of D and G colonies on agar, after periods of 24, 48, 72,
96, 120, 144 and 192 hours.   Considering the results as a whole it appeared that
the lower concentrations of peptone (0.2 and 1.0%), as well as the plain broth
control and the undiluted rabbit serum control, gave a small degree of dis-
sociation as compared with the high peptone concentrations (10 and 20%), as
recorded after a period of 72 to 192 hours.   In some cases the transformation
involved nearly 100% of the organisms after 96 hours.   But the important point
is that, after 192 hours, there sometimes occurred a change in proportions by
which the readings gave 50 D to 50 G—in other words indicating a regeneration
of the D type.   The same results are indicated in another experiment in which
it was shown that, starting with a pure type D culture in plain broth, the
type G organisms reached a maximum after 173 to 197 hours, but that the
relative number was lower after 228 hours and still lower after 18 days.   The
same was true of a culture of D in 5% serum broth.   In still another test, in
2% peptone solution, the count at 15 hours showed all D type, at 96 hours the
ratio of 40 D to 60 G and at 146 hours all D again.   In other words, in all of
these tests there seemed to occur, after the initial D → G transformation, either
a renewed overgrowth of D or a higher mortality among the G forms, which
would be unusual.   De Kruif presents no data that permit us to decide between
these alternatives.
    The second investigation mentioned above conducted by Morishima [352]
differed from that of de Kruif in that, instead of growing his normal culture
(B. typhosus) continuously in the same tube of serum broth, he transferred
from tube to tube daily over a considerable time; moreover Morishima

employed typhoid immune horse serum while de Kruif used normal rabbit serum. Since the further details of Morishima's tests have been presented in section 10, it is now sufficient to recall that, although the immune serum at first caused a rapid change of the normal culture to the nonagglutinating type (in homologous immune serum), continued transfers through the same medium caused, or permitted, a return after a varying time to the normally agglutinating culture. This seems to be equivalent to the $D \rightarrow G \rightarrow D$ transformation observed by de Kruif occurring in the same tube of medium after varying intervals. We note that Feiler on the contrary was able to carry his strains of nonagglutinating typhoid bacteria (also produced under the influence of immune serum) through 174 consecutive immune serum broth passages without any tendency to reversion. Indeed, when this culture was returned to normal medium (serum free) the condition persisted for 18 to 25 generations. These results of Morishima are difficult to explain and seem to uncover a new question for which, at present, we have no answer. There is the possibility, however, that the transformation first observed under the influence of the immune serum was not the $S \rightarrow R$ transformation, but only the $S \rightarrow O$, due perhaps to a weak immune serum. In this case the continued cultivation under the same conditions might have served to complete the dissociation to the R type. Unfortunately, Morishima did not present cultural or other data which would make an opinion on this point possible.

The Nature of "Reversion."—In concluding this section dealing with the so-called permanence of the R type, one further aspect of the problem demands attention. In our consideration thus far of the retransformation from R to S, I have employed the term "reversion," since this has been in common use in the literature and seems to cover the sort of change superficially observed in the cultures. I believe, however, that we shall do well to consider very carefully another possibility: Is the reverse transformation from R to S in reality a reversion or is it a further *progression?* In dealing with this question, which I have not found treated in the literature, although it is suggested in the work of Mellon [331] on B. coli mutabile (with reference to cell forms and their fermentation reactions), I wish first to present my own view of the nature of the changes. We may then undertake to ascertain what evidence supports this view.

Experimental data already presented with special reference to the serologic and cultural (colonial) aspects of dissociation indicate, for several bacterial species at least, three distinct antigenic components, S, O and R. Of these there can be little doubt in the cases mentioned; whether there are still other components must remain a question. These antigenic components are found united into various, but fairly orderly, combinations so as to produce culture forms (antigenic configurations) which have also become recognized as S, O or R cultures, named (according to one scheme) from the particular antigen that is most

prominent. It does not need to be said that these antigenic configurations are found to be correlated with certain culture types of fairly distinct morphologic characteristics. Although some cultures exist which apparently contain only a single antigen (O or R), they more commonly contain two; and it is possible that all three may be present in a single culture. Accepting therefore the fact that the serologic culture type is determined by the nature and balance of the antigenic components which make up the configuration, we may make use of capital letters to designate the antigen which is dominant in the configuration, and of small letters to indicate the antigen that is commonly secondary. Making use, then, of the experimental data supplied by Weil and Felix, Arkwright and Goyle, White, Goyle independently, and Baltenu, as well as of many incidental observations made by others, we may set up the antigenic constitution of the three chief culture types as follows:

S type (normal smooth) becomes...... So
O type (smooth variant) becomes..... Or (or O) (or sO)
R type (rough variant) becomes...... Rs (or R) (or oR)

In other words, the S type culture always contains some O antigen, although the S is dominant; the O type culture is likely to contain some R antigen, although the O is dominant; and the R type culture may contain some S antigen, although the R is dominant. With respect to the antigenic configuration of the R type culture, White [487] (p. 80) expressed the view, based on absorption tests, that rough cultures often show a sufficient remnant of O antigen "to absorb all or nearly all the smooth O agglutinin from a smooth serum." In addition, Goyle has pointed out that both O and R culture types may appear which are apparently pure O and pure R, respectively. It might happen that the "pure R" antigen is correlated with those culture forms which show the often described, marked constancy in their adherence to the R features. The normal S type culture, on the other hand, seems never to consist of pure S antigen; it always contains O. That it sometimes may also contain R antigen (especially in old cultures) seems probable from results of cultural and serological tests, and especially in those cultures where the O form rapidly gives place to the R, as in the dissociation of the pneumococcus and Bact. lepisepticum. The points thus far presented are strongly supported by experimental evidence from B. typhosus, B. enteritidis, B. dysenteriae, B. cholerae suis and (to a slighter extent) from B. proteus X19.

The Trend of the Dissociative Reaction.—The question now arises regarding the trend of the dissociative reaction, starting from the "normal" type S culture. As has been shown by considerable evidence, although the generation of the R type culture often seems highly abrupt, it is in many cases a gradual process, and the "extreme variant, R" is not ordinarily attained until a considerable time after the first rough characteristics may be noticeable in the colony or culture. Moreover, we have seen that in many instances an intermediate or transitional form of culture intervenes between the S and the R forms, although in certain cases this intermediate seems to be absent. Even here, however, we have noted that there is reason to believe it still exists, but for some reason that is still beyond us fails to make its appearance on the usual culture mediums. This is what we have designated the O type, although it would be better to say, one of the O types (intermediates), since they comprise a somewhat variable group, and it is only certain members of this group that are the most unstable. This aspect of the matter has already been presented in section 6 and it is unnecessary to repeat it here. Although the transitional form is apparently lacking in some instances, in others, as in B. proteus for example, the transforming organism seems to halt easily at the intermediate stage and to demand a greater stimulus to force it over to the R form. We might perhaps anticipate that different species would vary in the readiness with which they become transformed into the "extreme variant." Considering all the data at hand, the direct transformation from S to R, however abrupt it may appear, is highly improbable.

The establishing of an intermediate type of culture, characterized by a specific antigenic configuration, has therefore the great importance of determining a *direction of transformation*. The actual process of change is, therefore, not S → R, but S → O → R. The first phase of this transition (S → O), as we are led to believe from the work of Arkwright and Goyle, White, Goyle and Balteanu involves a loss of the heat labile antigen (S), and an increase in the heat stable antigen (O), which we know is always present to some extent in normal S cultures. At the same time the configuration may perhaps gain a little R. The configuration therefore changes from So (normal culture) to O or Or (intermediates). It would seem probable that sO also exists at a certain stage in transition, but this has not been reported. In any case, the S antigen has been largely lost by the time the intermediate O culture type has been reached. Among the many and various cell forms that

comprise the O type culture, just which one, or ones, are responsible
for the marked antigenic differentiation cannot be stated, but establishes
an interesting problem.

But many observations already cited show that the intermediate type O
culture, when compared with the S and R cultures, is more unstable. It
can either revert to the S culture or progress to the R, depending no
doubt upon whether it has lost all of its S antigen and upon the nature,
intensity and duration of the stimulus that forces the reaction. Indeed,
these O type cultures seem to be "opportunists" insofar as, within the
limits of their antigenic structure, they are quick to seize upon any
advantage offered by changing environment or circumstance. If the
conditions provocative of dissociation are maintained or intensified, the
transformation to the R type culture sets in, the R antigen being
acquired by degrees. The type O culture thus changes from O, or Or
(according to White's data), to oR or R.

Whether the culture now remains in the R state or begins to generate
the S antigen, as seems to be the case with many partially stabilized R cul-
tures, again seems to depend on the environmental conditions. We have
already examined some instances in which great stability has obtained;
others in which the retransformation to S culture has occurred within
a short time. In any case, whatever remnant of O antigen may have
remained in the R configuration is dropped and some S antigen is
gained. The configuration accordingly changes from oR or R to Rs,
which culturally at least may still be a typical "rough." If the "rever-
sion" is destined to complete itself, the culture passes through rS, not
apparently to S (pure), but to So, representing again the normal
S culture type. And thus the antigenic cycle is completed. Whether
there may exist a transitional stage between R and S in the R → S
transformation, such as is found between S and R in the S → R trans-
formation, cannot yet be stated. It has never been described, but its
existence seems possible. Even if found, however, it is doubtful if a
new antigenic group would be added to the three types already indi-
cated. These three, independently or in combination, provide ample
basis to afford a serologic differentiation of the three chief cul-
ture types already considered. On the other hand, it seems probable
that, under varying conditions of cultivation, there may exist still other
antigenic configurations incorporating the three essential antigens, or
any two of them, in varying degrees, and which would be represented
as other arcs about the central antigenic circle. It is clear, however, that

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for some unknown reason the chief culture types become stabilized near to the three antigenic points. These also mark a stabilization with respect to the colonial and cultural characteristics of the three chief types, as has already been shown.

The Antigenic Cycle.—The preceding exposition illustrates my meaning when I have said that it seems most likely that the R → S transformation takes place, not through true reversion, but through further progression; and thus effects what I have termed an antigenic cycle, rather than a "back-and-forth" variation. Any final conclusion that such a cycle exists requires more and better evidence than is now at hand in the literature bearing on dissociation; but many circumstances point strongly to such a cycle of change involving the main culture types and their varying antigenic constitution. Some of these points may be briefly referred to:

While cultural examinations, carefully made, have usually demonstrated the O type intervening between the S and R types in the S → R transformation, I know of no evidence to indicate the intervention of this type, or of any other type, in the R → S transformation. (But see Soule,[450] P type.)

At the very beginning of dissociation, in nearly all cultures that have been examined carefully, the first sign of antigenic change involves a loss of the S antigen and an increase of O antigen. The second stage involves the loss of O antigen and the gain of R. The third stage involves the loss of R antigen and the gain of S.

If the R → S retransformation were in the sense of a true reversion, then the R antigen should first lose R and gain O; and next, lose O and gain S. There is no clear evidence that either of these changes takes place. The first sign of transformation in the R culture is not the gain of O, but the gain of S. Many R cultures (not the "extreme") contain S antigen, in addition to R; just as the normal S culture contains O in addition to S.

In old broth cultures consisting exclusively of R, and from which the transitional O has quite disappeared (although it was present earlier), the S type culture may sometimes be seen to regenerate in limited degree (Bernhardt), but still without the reappearance of the transitional form.

The conclusions to which I am thus led from the foregoing observations will doubtless remind the reader who is familiar with the valuable works of Mellon, Enderlein and others, of the cycles of development which they have postulated for various bacterial species, based largely upon the changes in the morphology and reproductive function of the individual cells. While "life cycles" of bacteria will doubtless eventually be worked out on this basis, I have for some time been of the opinion that, if a cyclical development is established, it will be first on a larger pattern than that cut out by the majority of recent writers, whose work (with the exception of Mellon's) has not

consulted in appreciable measure the fundamental antigenic and
serologic aspects of the transitions; and which has also per-
haps made too slight use of the striking cultural aspects which are
found to underlie the various culture types, the significance of which
we are just beginning to appreciate. In nearly all the published work
dealing with the alleged life cycles of bacteria I have found it a difficult
if not impossible task to ascertain what the definitely "cyclical" aspects
actually were, although the fact of transitions has often been clear
enough. And I believe that these cyclical phases will become fully
clear only when we grasp the problem with reference to the varying
antigenic and serologic features of the chief components—the general
nature of which, I believe, is now fairly well established. When such
fundamental data are fully in hand it will be both an interesting and
a necessary task to learn what the cell changes actually are that support
this diversified antigenic structure; also what relation these chief com-
ponents bear to the filtrable forms of bacteria which, despite much
current protest from the still surviving monomorphists, may now be
recognized with certainty in many bacterial species.

   Conclusion.—In concluding this consideration of the socalled reversi-
bility of the R type I quite agree with Baerthlein [30] that the possibility of
its demonstration will depend on the method employed. We know that
all cultures capable of manifesting dissociation are not made to show
the reaction by the same condition or environment, although there may
always be a common factor in such conditions, in that they are preju-
dicial to what we term "normal growth." It is legitimate to believe
that the conditions which enforce dissociation are not the same as those
which enforce or permit reversion; and many instances bearing on this
point have been presented in the foregoing pages. I believe, therefore,
that we shall scarcely be justified in the view that such reversion is not
to be expected in all cases until we have exhausted every means of
modifying the environment of the organism concerned. It does not
seem quite in accord with general biologic principles to assume that
variations of hereditary significance (that is, true mutations) would be
formed as easily or as commonly as we observe to be the case in the
production of cultures of the O and R types; or that microorganisms
in general are addicted to discarding permanently their ancient hereditary
characters with such apparent nonchalance as can be observed in the
frequent examples of the dissociative phenomenon occurring in every
laboratory.

13. DISSOCIATION DETERMINED BY THE LYTIC PRINCIPLE (BACTERIO-
PHAGE) AND SUGGESTIONS FOR A MODE OF REFERENCE TO
THE SECONDARY DISSOCIATES

Whatever may be the nature of the bacteriophage of d'Herelle, or of its lytic influence upon the bacterial substratum, the fact has become clear through numerous observations on many bacterial species that, when employed in weak dilutions, or sometimes in strong, it acts more or less like an antiseptic substance (phenol), like some dyes (gentian violet, malachite green), or like immune serum, insofar as it produces, or intensifies, culture changes which are of the general nature of dissociations: O types and R types of culture of various grades and potentialities are generated from the "normal" culture of the substratum. There is to be observed, however, between the action of the bacteriophage and other agents of dissociation, a marked difference in the speed of the reaction. Without relating them in any way to the dissociative process, or to any phenomenon allied with it, both Bordet [61] and d'Herelle [247, 248] have recorded the origin of "mutating" culture types under the lytic stimulus; and Bordet has gone so far as to set up the lytic principle as an agent which, through its power of effecting "mutations," acts as a "director of evolutional progress"; and in this way "controls the destiny of the species." It may be that the thing which we call the bacteriophage is so bound up with the "destiny of the species," but not, I suspect, in the manner in which Bordet believes. We have already considered the nature and effects of active microbic dissociation as it appears spontaneously, or as it is forced under the pressure of unfavorable environment. But no treatment of the problems of microbic instability would be complete if limited to this aspect of the matter. In the present section we shall therefore turn our attention to the dissociation of microbic species as determined by the influence of the bacteriophage; and we shall see that many features of transmissible bacterial autolysis, as the bacteriophagic phenomenon has been termed by Bordet, resemble closely those of microbic dissociation—not merely in the nature of the results effected, but also in the mechanism of the reaction so far as we are at present able to understand it.

Cultures Secondary to Lysis.—The subject may be made clearer if we first review the simple facts established by the studies of d'Herelle and of Bordet. When the bacteriophage in appropriate concentration is added to a young broth culture of the sensitive organism lysis takes place within three or four to 24 hours and most of the bacteria are apparently destroyed. But some clearly survive. After some days

these may give evidence of new growth and produce a fresh clouding in the medium. This represents the secondary or resistant culture. It is usually resistant to the same concentration of the same bacteriophage but not necessarily to a stronger concentration of the same (Bordet [61]), nor to another "strain" of the bacteriophage (Zdansky [495]). In other words, the resistance to the lytic agent is not absolute but relative, just as we have seen to be the case in the resistance of bacteria to antiseptic substances. As we shall note later, these secondary cultures arising after lysis differ in many respects from the original sensitive form (S).

Similar results in the production of secondary cultures may be obtained on solid culture mediums. If the concentration of the lytic filtrate is such as to carry only a few "lytic units" per loop of filtrate, and such a loop is streaked over the surface of an agar slant which has just been seeded with several loops of a young broth culture of the homologous sensitive organism, after incubation there will appear on the surface of the agar a continuous growth of the substratum culture, but it will be spotted with lytic areas, usually round and measuring from a fraction of a mm. to 15 mm. in diameter. Here at first there appears to be no growth. After a few days, however, there will often be seen on these patches a sprinkling of small colonies which grow only slowly. Most of these colonies also represent the secondary or resistant culture. It is resistant to the same concentration of the same lytic agent but not necessarily to a stronger concentration, nor perhaps to some other "strain" of lytic agent.

Among the colonies that arise on such bare areas on slants, or appear on plates streaked with mixtures of broth culture of the sensitive organism and lytic principle, there are likely to be several types. Some appear identical with the original sensitive strain and are round, regular and soft. These are infrequent. Others of different build are resistant to the lytic influence and "carry" the lytic agent into subsequent transfers. Still others are resistant to lysis but do not "carry" the agent in subculture. Whether these cultures are lysogenic, that is, will perpetuate the lytic action, can be ascertained by inoculating their filtrates into broth cultures of the homologous S organism and noting whether lysis or inhibition takes place. If the culture does carry the lytic agent it may be freed from it by appropriate methods of cultivation (Bruynoghe [77]); or by dilution methods (Bordet [61]). This results in a resistant but nonlysogenic culture. It appears further that the resistant character itself can be made to disappear by long cultivation on solid mediums (d'Herelle,[246] Bruynoghe). The lysogenic colonies are some-

times revealed by their irregular shape and ragged outline (Gratia [211]). When the resistant colonies are seeded to agar slants they give a slow, modified growth, always compact, sometimes mucoid and often hard and tenacious when old. Transferred to broth they always give the agglutinative form of growth like the R type from active dissociation.

Microscopically the cells of the resistant cultures differ widely from the original type. Although sometimes (especially in the mucoid colonies) giving filamentous and beaded forms, they most commonly present fore-shortened rods which frequently approximate the cocco-bacillus or even coccus type. This is true of B. coli, B. typhosus, B. dysenteriae and many others (d'Herelle [245]). Such cultures, which also possess modified biochemical reactions, may be remarkably persistant in their new form and sometimes, according to d'Herelle,[248] permanent.

What has been stated thus far relates to the action of the lytic principle on the "normal" sensitive culture. But we have seen that, through dissociation, the S and R types may be isolated and grown separately. What then is the relation of the lytic principle to these two chief dissociative types?

One might conclude from d'Herelle's [248] summary dismissal of Arkwright's [17] statement regarding the relation of S and R to bacteriophagic action, that no significant relation exists. But this would be very far from the truth as is made clear by certain studies of Gratia [211] and of Fejgin,[168] as well as by many still unpublished observations of my own. If Arkwright, as d'Herelle somewhat pointedly states, "has no idea of what the phenomenon of bacteriophagy really is," it is apparent that d'Herelle is equally lacking in understanding what constitutes one of the most obvious cultural distinctions in microbic dissociations, a subject which has received no consideration in any of his published works. The results of Gratia's [211] tests, the proper significance of which does not appear to be brought out in d'Herelle's analysis of them, agree to a considerable degree with my own findings with apparently analogous cultures and may be reported to make this aspect of the matter clear.

So far as I am aware Gratia was the first to report on the action of the bacteriophage on the well established S and R types separately. In this case they were the dissociates of a single-cell strain of a sensitive coli culture. The lysis of the S form gave the resistant SR, while the lysis of the R gave the resistant RR. Compared with the original S culture the type R culture was markedly resistant to the bacteriophage

and gave many more secondary colonies than the former. No difference in resistance between the SR and RR secondary cultures is reported. In my own study of this point I have found that when S and R cultures (Shiga) are spread on agar plates, the addition of either anti-S or anti-R lytic filtrate makes a much slighter impression on the R, while lysis and inhibition of the type S culture is marked. Fejgin [168] also has found certain R types of the Shiga dysentery bacillus resistant to lysis by the bacteriophage.

It would be a misapprehension, however, to understand that the average type R culture from active dissociation is not to some degree susceptible to bacteriophage action, and this point will be considered further in the latter part of the present section.

It was perhaps the chief merit of Gratia's contribution, however, to reveal the numerous departures from normal type that can be produced by properly graded doses of bacteriophage acting on sensitive culture. Dilutions 10^{-1} to 10^{-5} of filtrate lytic for B. coli were used against a 12 hour culture in broth and the mixtures plated. The results showed not only that the number of colonies obtained varied inversely with the amount of the filtrate, but also that the amount of filtrate determined within limits the sort of colonies, and subsequent culture. In general there were three chief types of colony: mucoid (rare), nonmucoid and regular, nonmucoid and irregular. The mucoid colonies were nonlysogenic and on further plating gave rise to opaque, translucent and mixed colonies. The nonmucoid, regular colonies occurred only in high concentrations of lytic principle and bred true. The nonmucoid, irregular colonies were determined by lytic principle in moderate concentration and were lysogenic, as this term is later defined.

It may also be observed at this point that Blanc [54] reported the transformation of normal B. coli culture into a form characterized by mucoid colonies resembling the Friedländer bacillus as a result of bringing filtrates of sewage (which we know contains abundant lytic principle) into action against normal coli culture. It is not necessary to go to this trouble, however, since sewage already contains large numbers of this form of coli which are easily demonstrable on Endo plates. Similar transformations of B. coli into a mucoid aerogenes-like organism under the influence of the lytic principle have been reported by Bordet and Ciuca; [62] and by Gory [203] (using tapwater presumably containing lytic agent). D'Herelle [248] also reports having observed similar instances and assumes that all these cases have to do with "mutations" determined by the lytic principle.

It is important to note in passing that these various cultural modifications produced by the lytic agent on type S culture are not (at least with one exception) wholly peculiar to bacteriophage action. The same trend of transmutation, often revealing forms that are fairly resistant to lytic action, appears in many quite normal cultures when they are aged, or when they are brought face to face with various kinds of unfavorable environmental influence. In this respect, therefore, the action of the bacteriophage is not so unique as d'Herelle maintains. Perhaps the most striking difference that obtains between the R series of active dissociation and the R series of transmissible autolysis is that, in the latter, the resistance to lytic influence is greater; and that some of the secondary colonies produced under the influence of bacteriophagic lysis are themselves lysogenic. Even the last exception, however, we may eventually find to be gratuitous, since it already begins to appear that certain type R or other colonies of the active dissociation series exert a marked repressive (and perhaps lysogenic) influence on the growth of the normal S culture.

In his theory of transmissible autolysis Bordet was quick to seize upon the above mentioned observations of Gratia, and to confirm them, as supporting his conception of gradations in susceptibility of different organisms in the same culture to the lytic action, a point which forms the keystone to his entire theory. Fortunately the fact cannot be questioned. From many other observations there can be little doubt that the extent to which a culture proves resistant to lytic action, as also the extent to which it is capable of elaborating additional principle, depends not only on the strength of the lytic filtrate applied, but also—and perhaps primarily—on the state of the culture, the degree to which dissociation has progressed at the moment when the lytic test is performed. This, in turn, will depend upon the ability of the culture to undergo dissociation and may also be a function of time.

In connection with the forms of culture resistant to lysis by the bacteriophage, their origin and mode of generation is of interest; and especially d'Herelle's conception of these phenomena. D'Herelle's view is indicated by the following statement taken from his latest book [248] (p. 189). "The secondary cultures, then, are the result of the adaptation undergone by the bacterium which acquires an immunity to its parasite." He points out that the nitrate and acetate of lead, also nitrate and sulfate of silver, added to the culture favor the reaction. Aside from such stimuli, secondary cultures "have their origin in the operation of the phenomenon of natural selection whereby some bacilli show a

greater aptitude than others to the acquisition of resistance to the bacteriophage" (p. 191). Although Gratia [211] had suggested on the basis of his own experiments that resistance of cultures to the bacteriophage might not involve the gain of resistance by the bacteria (after put into contact with the bacteriophage) so much as a selection of those organisms possessing a natural resistance (previous to contact with the bacterio-phage), d'Herelle cannot accept this view, since for him the bacteriophage always acts on a "normal," homogeneous culture. For him selection operates, not on bacteria naturally endowed with greater resistance, but "through a selection of those susceptible bacteria which are more apt at acquiring resistance" (p. 191). In examining d'Herelle's position on this point it becomes clear that his objection to Gratia's view and all similar views of a previously-existing resistance to lytic action on the part of certain cells of the culture, is based on a lack of knowledge, or appreciation, of the process of dissociation of a normal culture into a culture form (R) more resistant to the action of the bacteriophage. The experiments of Gratia and my own are in agreement in indicating that the cells which are resistant to the lytic agent of medium strength are identical with those cells which comprise the R culture type, often mixed with the S forms. Their resistance to the bacteriophage is pre-determined in the cyclogeny of the species; it is not produced de novo by the bacteriophage, although certainly in the course of bacteriophage action the resistance may become intensified. This is, I believe, the true explanation of the circumstance that, when an anti-S type bacteriophage is brought into action against an S type culture and an R type culture, the number of secondaries will be much greater in the case of the latter.

With further reference to d'Herelle's failure to comprehend the relation of microbic dissociation and of the R type culture to the phenomenon of transmissible autolysis, one other matter of considerable significance may be introduced at this point. The problem at issue is revealed by the following paragraph appearing in d'Herelle's latest book (p. 236):

"Strains contaminated by a bacteriophage may be recognized in that they give mutant colonies, while all the colonies of an ultrapure strain are identical. The bacteria of contaminated strains are but slightly, or not at all, agglutinable by a specific antiserum. The bacteria are resistant to the action of bacteriophages which attack homologous strains. Every time that one or the other of these characters is encountered there is reason to suspect contamination by a bacteriophage."

Nothing could be further from the actual truth than the information conveyed by this paragraph. Its composition was manifestly influenced throughout by the old, monomorphic conception of the nature of bacteria, which more than once serves to produce entanglements in d'Herelle's exposition of the bacteriophage and its mode of action. We may profitably analyze the paragraph mentioned by sentences. "Strains contaminated by a bacteriophage may be recognized in that they give mutant colonies while all the colonies of an ultrapure strain are identical." This statement is contrary to recognized facts because, as we have amply observed in the data on colony variation presented in earlier pages of this work, colony variability is one of the most fundamental and constant characteristics of any "normal," pureline, bacterial species; and, in the majority of cases certainly, there is no evidence that the bacteriophagic phenomenon, at least in the d'Herelle sense, is attendant. Evidence for this does not need to be repeated here. We may take the next sentence: "The bacteria of contaminated strains are but slightly or not at all agglutinable by a specific antiserum." This statement is true; but the circumstance is neither differential nor distinctive. While it is a fact that cultures resistant to the bacteriophage are only slightly agglutinable, or nonagglutinable in specific antiserum, it is by no means cultures alone which have proved resistant to lytic action that possess this characteristic; for it is also possessed by the R dissociates of all bacterial species that have been studied from this point of view. Neither the occurrence of spontaneous agglutinability nor failure to agglutinate with specific serums can thus be taken as a criterion of "contamination" with a bacteriophage in the d'Herelle sense.

Regarding the third sentence—merely because a culture is resistant to bacteriophages which attack homologous strains is in no way an indication that such a resistant strain is "contaminated" with lytic principle; and for the reason that any "normal" S type culture may dissociate into the R form and become more or less resistant to lytic action, even while homologous cultures, which have not undergone dissociation, are still sensitive to the lytic action. Finally, with reference to the last sentence: "Every time that one or the other of these characters is encountered, there is reason to suspect contamination by a bacteriophage." If this statement were true one would need to suspect the presence of bacteriophage in every sensitive (S type) culture capable of dissociation into the R state (which as we have seen is always endowed, among other characters, with exactly those features which d'Herelle states denote the contaminating presence of the bacteriophage). As a matter of fact,

I suspect that the lytic principle is present, potentially at least, in every such culture; but certainly not in a form that is within the scope of d'Herelle's meaning when he employs the term, bacteriophage. In every bacterial species thus far studied from this viewpoint there are stages highly susceptible to bacteriophage action, but other cyclogenic states in which the culture is much more resistant. Furthermore, when adequate tests have been made, it will undoubtedly be ascertained that there exist still other stages in the cyclogeny of a species in which the culture is actually refractory. These circumstances constitute added evidence of the close parallel, at least with reference to one important feature, between microbic dissociation and transmissible autolysis, a subject to be considered in greater detail in the following section.

Filtrable Forms of Bacteria Secondary to Lytic Action.—We have now observed the influence of the bacteriophage in producing modifications in normal, sensitive culture and in normal cells, as also in "pure" S and R strains. We may now turn to its influence in causing the generation of filtrable forms of bacteria. In the sections dealing with active dissociation in normal cultures it has been noted that such cultures may enter a stage of development in which they are filtrable through Berkefeld or Chamberland candles that are able to hold back all microscopic forms. In most of these instances no signs of dissociation have been noted at the time. It is also clear, however, that accompanying that form of dissociation stimulated by the bacteriophage, there often occurs a quick development of filtrable bodies. In 1922 d'Herelle[246] reported filtrable forms of the Shiga bacillus occurring in the filtrates of lysed cultures. Filtrable stages of B. coli, B. typhosus, B. dysenteriae and M. aureus under the influence of weak (homologous) lytic filtrates have been reported by Hauduroy[234, 235] and by d'Herelle and Hauduroy;[249] also for B. coli by Tomaselli.[463] Some of these filtrable forms were reported to yield a faint, opalescent growth in broth; and less frequently a delicate growth on solid mediums (Hauduroy). It seems also that they may propagate in the invisible state. In still other instances they may revert to the original, but still resistant, cell type; or to a modified cell type (coccoid). In such cases the opalescent growth gradually gives place to a definite turbidity. One point of interest in these cases is that, whatever the morphologic type of organism submitted to the lytic action at the beginning—rods or cocci—all show the same disintegrative trend toward granule formation; and in this end-state all meet on a common morphologic footing (d'Herelle and Hauduroy). In other words, the organisms concerned seem to

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have entered into a sort of morphologic cosmopolitanism, comparable with the "serological cosmopolitanism" of Schütze, relating in this instance not to the SR, but to the R types from microbic dissociation. I believe that we may eventually find in this "cosmopolitanism" or convergence of the rough types, whether morphologic or serologic, and whether occurring in connection with simple dissociation or with transmissible autolysis, a deeper significance than now attached; and one bearing upon a number of at present inscrutable problems recently introduced into bacteriological literature. What relation, if any, these and similar observations on bacterial convergence may have to the well established bacteriophagic heterogeneity of action in inhibition and lysis, remains to be ascertained. In this connection I have already shown,[227] however, how closely the bacteriophagic relations follow the serologic in the curious relationships existing between B. typhosus and the avian paratyphoids, Bact. pullorum and B. gallinarum. This phase of the subject is considered further in the following section.

But, to return to the subject of the filtrable forms of bacteria, it may be noted that Bronislawa Fejgin[171] has obtained a filtrable stage of the typhoid bacillus capable of producing an experimental disease in guinea-pigs. The virus was obtained through the action of a weak lytic principle on normal typhoid culture. From her results Fejgin concluded that "the invisible virus of typhoid is only the bacillus of Eberth lysed by the bacteriophage of d'Herelle." In a later communication Fejgin[171a] reported that, from the brains of guinea-pigs infected with typhoid virus, she could obtain by cultural means minute coccobacilli which differed from the normal culture biochemically and serologically, and which she believed represented the formation of a microscopically visible secondary culture in vivo.

By the use of a lytic principle acting on proteus X19 Fejgin[169] was also able to produce a secondary culture resembling in many respects the "natural" OX19 strain of Weil and Felix, as well as the O forms produced artificially by Braun and Schaeffer by resort to phenol and to "starvation agar." Thus, in the case of B. proteus, we observe that essentially the same results have been secured (with respect to obtaining dissociation) by five distinct methods: spontaneous dissociation; reaction to phenol; reaction to starvation; heating, and reaction to the lytic principle. Here again, therefore, the bacteriophage is not unique in its ability to produce "mutations." The essential phenomenon involved is microbic dissociation, but the fundamental reaction on the part of the bacteria may be incited in various ways.

In concluding these references to the filtrable forms of bacteria, generated under the influence of the lytic filtrates, one further allusion is required. Insofar as these filtrable particles not only survive the action of the lytic principle, but also propagate in a favorable medium (Hauduroy [235]) they might be regarded as a sort of secondary, resistant type (d'Herelle and Hauduroy [249]). If, as these workers also assume as a result of their more recent experiments, the lytic principle is bound up with some of these filtrable forms, as well as with intact organisms of the resistant type, producing a symbiosis (d'Herelle and Hauduroy), such filtrable forms would also be regarded, in the Bordet [60] sense, as lysogenic. Only a beginning of their study has as yet been made.

The Antigenic Relation Between R and SR Cultures.—The characteristics of the secondary cultures arising after lysis of the S type may also be considered in relation to serologic reactions. Few studies on this aspect have been conducted but those that exist afford interesting data. They may be considered especially with reference to the agglutinative and protective reactions.

It has already been indicated that when an R type culture is injected into rabbits the serum is likely to contain agglutinins for the R type, but not (or not strongly) for the S. The R form is different antigenically. The question naturally arises regarding the antigenic relation between the S type and those cultures that arise as secondary to the action of the bacteriophage. This point has been partially studied by McKinley.[324] Rabbits were immunized against the original culture (coli S); and against coli resistant to lytic principle (SR). Each serum agglutinated its own antigen but not the heterologous antigen. The complementary point, which is quite important, but which was not within the scope of McKinley's tests, is whether there exists an antigenic difference between cultures of the R type and cultures arising as secondaries to lytic action (SR). Also whether there exist reciprocal antigenic relations between various lytic secondaries, as Arkwright found there existed close antigenic relationships between the various R types of dysentery Shiga; or whether there are such antigenic differences as Felix [172] found to be the case in the "Zwischenformen" of B. proteus X19. Of the fact that both microbic dissociation and transmissible autolysis involve the generation of antigenic "mutants" there can be no doubt; but are the transformations along the same line of antigenic modification? This point is of much importance in any detailed comparison of the dissociative and autolytic reactions (section 14). No data bearing on this subject are yet available.

The Nature of the S → SR Transformation.—Regarding the nature and significance of the change from the normal to the resistant type under lytic stimulus, d'Herelle,[246] in the earlier stage of his investigations on the bacteriophage, was inclined to regard these changes as due to the effects of natural selection, by virtue of which only those bacteria most resistant to the bacteriophage were able to survive; and they survived, according to d'Herelle, because they were able to undergo adaptation: "Secondary cultures, then, have their origin in the operation of the phenomenon of natural selection whereby some bacilli show greater aptitude than others to the acquisition of resistance to the bacteriophage." In this reference it will be clearly seen that there is no allusion to actual transmutation in the ordinary sense. The result was due merely to a survival of some cells among fluctuating variations that existed pre-formed in the original culture.

But this point of view was apparently unsatisfactory to d'Herelle, for, in his second book,[247] he lays stress on the fact that some of the cultural modifications observed in bacteria under the influence of lytic principle actually possess "mutational" significance. Indeed, in his second book d'Herelle gives us to understand that "these mutations are always produced under the influence of the bacteriophage," a view warmly seconded by Bordet.[61] In 1926 d'Herelle's [248] belief in the mutation-producing power of the lytic agent is undiminished for he states in his last book, "It is indeed probable, as various investigators have suggested, that all of the fixed mutations occurring among bacterial species are produced through the action of the bacteriophage." This naturally suggests that no "mutations" can arise in bacteria, purely from intrinsic causes, or from extrinsic causes other than the bacteriophage; and that this is actually d'Herelle's view may be inferred from his further statement, "a pure bacterial strain is not subject to transfor-mations." In view of our present knowledge of the facts regarding microbic dissociation, even if the explanation is not in hand, we can scarcely accept the truth of either of these statements. Not only pure bacterial strains, but even pure line strains, are certainly subject to transformations of a very far-reaching and remarkable sort. Moreover, although we cannot fail to recognize in the lytic principle an important agent in the production of so-called bacterial "mutations," we cannot safely regard it as the only factor; nor even assume that, in its essence, it is a fundamentally different factor from some others already at work in producing the phenomenon which we have termed "active

microbic dissociation." To this subject we shall return in a following section.

Terms of Reference to the Secondary Dissociates.—The important point which the above findings bring out, however, is not that cultures dissociate into the S and R types, and that it is possible to obtain a "strain" of bacteriophage for each of these dissociates, but that, when such dissociates in turn undergo transmissible autolysis, each gives its own type of secondary and that these in reality appear to form a continuous series of transformations from the original type to the new "mutant." The transitional types punctuating this series are determined by the nature of the original culture and the strength of the lytic stimulus. Just as we may term the cultures arising from active dissociation the primary dissociates, I propose for these new cultures (arising as secondaries from bacteriophagic or passive dissociation) the secondary dissociates. To what extent these two forms of the culture resemble each other, and to what extent their generation may be dependent upon similar forces, will be considered later. The situation, however, is one that demands for purposes of clear exposition an expansion of the terms of reference already proposed. The following suggestions can perhaps be followed most clearly by the employment of concrete examples. The scheme presented has an experimental background in the studies of Bordet, d'Herelle, Gratia and Zdansky, together with some observations of my own on the behavior of the R types of culture under progressive lytic stimulus. I therefore suggest the following terms and, to make the subject clearer, I reintroduce the terms already proposed for the primary dissociates occurring in the primary reaction.

TERMS REFERRING TO THE PRIMARY DISSOCIATES

Type X. Refers to an original culture from a natural source. Likely to resemble the following type S but with somewhat different serologic reactions.

Type S. Usually a "normal" culture such as the average laboratory stock in the case of saprophytic bacteria. Contains besides antigen S, some of the antigen O of the "intermediate culture type"; if an old culture, it also contains some R. The normal, virulent type in acute infections. Exception in case of B. anthracis.

Type O. Representative of the intermediate or transitional group of cultures. Sometimes contains besides antigen O, remnants of S antigen and often some R antigen. Usually unstable culturally, and can transform either to S or to R. Probably the active culture element in the "suicide cultures" and in the S cultures in the lytic state ("Ly" of this list). Its natural end product in further dissociation is the R.

Type R. A dissociate from S through the intermediate O. Found particularly in old laboratory cultures of saprophytic bacteria and sometimes comprising the chief element in laboratory stocks of pathogenic organisms not maintained on fresh blood or tissue mediums. Contains R and often some

S antigen. A natural product of the influence of homologous immune serum. Especially stable and often reported permanent, but commonly transforms to the normal S.

Type Ly. Superficially an S type culture in the active state of dissociation (acute lytic tendency); actually an S type culture transforming rapidly to the intermediate O, with the rapid dissolution of this culture form, leaving as its product the R. In all but suicide cultures the lytic state can be perpetuated easily by transfer. Plating yields S, R and (sometimes, but not always) Ly colony forms. The Ly colonies are probably identical with what has been described as the "third colony intermediate." May also be slightly lysogenic.

Terms Referring to the Secondary Dissociates

Type SR. The secondary and somewhat resistant culture arising from the action of a lytic filtrate (bacteriophage) on its homologous substratum of the S type. Fairly stable. Plating gives mainly SR together with some S forms. Common in chronic and carrier infections in which lytic principle is also present. Antigenic configuration unknown.

Type RR. The secondary and resistant culture arising from the reaction of the lytic principle on R type culture. Equally or perhaps more stable than SR. Plating gives mainly RR forms. Usually a laboratory product. Antigenic configuration unknown.

Type Lg (lysogenic). An SR or RR culture resistant to lysis but which "carries" the lytic agent. Filtrates may initiate serial lysis in the homologous S substratum. Probably stable for many generations except on glycerin agar or sugar media. Plating gives SR or RR and Lg forms, but probably no S. Antigenic configuration unknown.

Types SR^2, SR^3, RR^2, RR^3, etc. SR and RR cultures of increasingly greater resistance to the lytic principle. Correspondingly increased stability. Usually laboratory products.

Types SR^n, RR^n. SR or RR cultures possessing maximum resistance to the influence of the lytic principle. An hypothetical type representing the most resistant, and pari passu, most stable, form of R, possessing "absolute" resistance to the lytic principle.

Resistance to the Lytic Principle is Relative, not Absolute.—No special reference need be made to these various culture types except perhaps in the case of SR^2 and SR^n. As is now well known, many cases of cystitis and pyelitis or pyelonephritis are due to infections with B. coli. From such cases the causative agent can be isolated and in a certain number is accompanied by a lytic agent present in the patient. Although one may suppose that the original infecting agent was an S type coli, an organism of this sort is seldom isolated from the urine; it is sometimes an R type, but when lytic principle is also present, it is an SR or an RR culture. The same may be true of some chronic typhoid infections, particularly urine-carriers. Two such carriers which I have examined showed SR or RR types, another showed only the R type. In such cases (presence of SR or RR) we are therefore confronted with the incongruity of a strong but subacute infection existing in the face

of a lytic principle sufficiently powerful to destroy laboratory strains of typhoid culture, but quite powerless to cope with the case strain.

Even under these circumstances, however, as Bordet [61] has shown by in vitro tests, and as Zdansky [495] has shown in clinical tests, we do not need to conclude at once that the case strain of B. coli or B. typhosus maintaining the chronic infection is not susceptible to lysis. It is merely required to discover or to produce another lytic agent possessing greater capacity to attack the SR or (probably less often) the RR culture. Such effective strains may usually be found in mixed city sewage as pointed out by Zdansky and confirmed by myself. Experiments involving this point have more recently been performed by Bordet [61] under well controlled laboratory conditions and using, not different lytic agents, but different strengths of the same lytic agent. Bruynoghe [77] has also demonstrated the wide range of resistance among the cultures secondary to lytic action.

But the circumstance that an SR or RR culture, resistant to one lytic principle, can be influenced by another or by a stronger one indicates to us that the resistance of the SR and RR types is not absolute or final. It is merely a relative resistance; for when tests are performed in vitro, we may find further resistant strains arising from these already somewhat resistant substrata after the application of the second or the stronger lytic filtrate. Such cultures are, in a way, "super-resistant" and accordingly may be designated SR^2 or RR^2 depending on their origin from S or R. Whether such cultures are still susceptible to another lytic agent cannot at present be stated. It seems probable, however, that the time arrives when no discoverable lytic agent has the power to effect further dissociation in the super-resistant culture; and this hypothetical "ultra-resistant" form might be termed the SR^n or RR^n. Its actual existence is merely postulated, for experiments leading to its demonstration have never, to my knowledge, been performed.

Regardless of the existence of such R^n types, however, present evidence is sufficient to demonstrate that the so-called "mutational" progress as "dictated" by the lytic principle is cumulative, just as we have found that the development of the R types of normal active dissociation is cumulative. High resistance to transmissible autolysis is seldom if ever gained in a single "stepping-up," except perhaps under the influence of a lytic agent of extreme energy; and under these circumstances the result is more likely to involve the complete disappearance of the bacterial culture than to make possible an adaptation.

Conclusion.—Many of the matters presented in this section are of much interest and significance in the formulation of any theory of transmissible autolysis; and the omission of several of them in d'Herelle's various expositions of his theory of the nature of the bacteriophage constitutes a failure to establish defense at one of its most vulnerable points. Although d'Herelle, at the time of his earlier studies,[246, 247] was able to recognize differences in the relative numbers of secondary colonies that arose from lysed-out cultures on solid mediums, and noted that old cultures, particularly broth cultures, gave more secondaries than young cultures, he apparently did not recognize the phenomenon of microbic dissociation, appreciate its significance in the production of "mutations" nor understand its influence in determining, in part at least, the varying degrees of sensitiveness or resistance to lytic action. If, however, we "read between the lines" of many of his experimental reports, we can observe the influence of the R types in determining the results; and experimental evidence for this has been presented particularly by Bordet[61] and Gratia.[211] D'Herelle's chief mischance in connection with this phase of his subject lies in the circumstance that he has attempted to understand and to depict the causes and trends of "pathological" variation and autolysis before he had adequately surveyed the nature and range of the "normal," cyclogenic variations involved in dissociative behavior. It may be that the keystone to the grand structure that d'Herelle has reared about the bacteriophage lies in his statement, already quoted, "a pure bacterial strain is not subject to transformations." If this stone crumbles, the edifice can no longer stand, at least without far-reaching reconstructions. We have here touched on perhaps one of the most important aspects of the dissociation problem, and one to which we shall return in a subsequent section.

14. COMPARISON OF THE MECHANISM AND RESULTS OF ACTIVE
MICROBIC DISSOCIATION WITH THOSE OF TRANSMISSIBLE
BACTERIAL AUTOLYSIS (D'HERELLE PHENOMENON)

Although it is not primarily within the scope of this review to consider the nature of the bacteriophage or the mechanism of transmissible autolysis, the circumstance that both of these phenomena appear to play so important a rôle in microbic instability, and that both involve cultural reactions and other modes of expression that are, at least superficially, so much alike, makes it impossible to omit a special reference to certain points of similarity as revealed in more recent literature.

The only writers who, to my knowledge, have referred to any distinct points of similarity between the phenomena of what we have come to term microbic dissociation and the phenomenon of transmissible autolysis are Eastwood [144] and Hoder.[253] Eastwood's conclusions of his brief review may be put in his own words. After noting the confusing mass of data to be explained, he states: "It would simplify matters greatly if one could recognize some general principle underlying this general confusion and could attribute the different types of variants to different phases in the operation of this principle."

And again: "In the search for a common factor, it is observed that these changes only occur with living and actually growing bacteria and that, therefore, they are probably due to some influence which operates at the nascent stage of growth. Hence the common factor can only be explained in terms of vital processes; but about these no precise information is available."

In attempting to formulate a general explanatory statement, Eastwood succeeded in pointing out merely that bacterial protoplasm has two functions—catalytic and synthetic; and that any disturbance of the balance (which is very minutely adjusted) will tend to the production of variants. He did not advance toward the fundamental facts of dissociation.

Hoder apparently noted clearly the similarity between "bacterial mutations" and the secondary cultures arising from lysis by the bacteriophage. He concluded that the same sort of influence or injury which brings about the one also produces the other. He accepted this circumstance as favoring Bail's [33] "splitter" or "chromosomal" theory of transmissible autolysis but did not recognize the phenomenon of dissociation as underlying the "mutations" observed.

It thus appears from the literature that practically no far-reaching attempts have been made to investigate the relation that actually exists between microbic dissociation and transmissible autolysis. Even those that have indicated a partial relation with mutation phenomena have not penetrated beneath the superficial aspects of the reaction. For this reason we may now attempt to ascertain for ourselves to what extent there may exist analogies between these seemingly quite distinct phenomena.

Relation of Active and Passive Dissociation with Reference to Incidence.—A survey of the bacterial species that have been observed to manifest noteworthy dissociative reactions and a similar survey of the

species for which a lytic principle has been obtained, reveals a striking fact. Although there are some species showing active dissociation that have not yet been found susceptible to bacteriophage action, I know of no species clearly susceptible to the bacteriophage that has not also revealed, and in a particularly striking manner, the phenomenon of active dissociation. Moreover, a further study of the literature makes it apparent that those bacterial species most commonly and most easily used for an elaboration of a specific lytic principle are, without exception, the ones that most easily manifest microbic dissociation. Indeed, our present conception of this phenomenon has been built up to a large extent on the strength of data derived from B. coli, B. typhosus and B. dysenteriae Shiga; and, as is well known, it is precisely these organisms that have served as the ground work for the huge structure that d'Herelle has raised in his study of the bacteriophage and its behavior, as well as for the greater part of all subsequent study on this phenomenon by others. Practically all of Bordet's studies have been based on that classical "mutating" form, B. coli.

In further reference to the above statement I would mention especially the following bacterial groups:

Colon-typhoid-dysentery.
Paratyphoid-enteritidis.
Avian paratyphoids: (Bact. pullorum, B. gallinarum).
Capsulated: (Friedländer's pneumobacillus).
Pasteurella:[1] (B. pestis, Bact. lepisepticum, B. cavisepticus, B. bovisepticus).
Suppurative: (Micrococcus albus, M. aureus, M. citreus).
Proteus:[2] (B. proteus).
Moro-Tissier:[3] (B. acidophilus).
Diphtheria:[4] (B. diphtheriae).
Asiatic cholera;[5] (Vibrio comma).

[1] In the Pasteurella group dissociation has been observed by Gotschlich,[204] Dudschenko[137] (B. pestis); by Bernhardt[49] (B. avisepticus); by Bernhardt[49] and de Kruif[119] (Bact. lepisepticum). A lytic principle for B. pestis has been obtained in 1923 by Villason[474] and in 1925 by d'Herelle;[246] for B. bovisepticus by d'Herelle.[246] The B. pestis caviae for which Bronfenbrenner[73] has isolated a lytic principle is not a member of the Pasteurella group.

[2] It is certain that Weil and Felix,[483] Baerthlein,[27, 30] Braun and Schaeffer[66] and others were dealing with an actual dissociation of B. proteus X19. I have been able to dissociate a laboratory strain of proteus. A lytic principle has been obtained by d'Herelle[243] and by Fejgin.[169] I was unsuccessful in an attempt to obtain from sewage a lytic principle for this culture.

[3] In this group a lytic agent has been reported by Sierakowski and Zajdel.[412] In two attempts I have not been able to confirm this, but I have obtained the dissociation of a culture of this organism isolated from a carious tooth.

[4] The dissociation of B. diphtheriae has undoubtedly been observed many times, most recently by Crowell.[114] I have observed the dissociation of the "Park 8" strain. A lytic principle has been reported by d'Herelle;[248] also by Fejgin[170] and by Blair.[51]

[5] The dissociation of this organism was probably observed by Baerthlein,[30] and more recently by G. Petrovanu[383] in 1924. The latter also succeeded in obtaining a lytic agent against the S, but not against the R type culture. A lytic principle was also reported by d'Herelle,[246] Jötten,[275] Meissner[320a] and by Flu.[181]

Streptococcus:[6] (Streptococcus hemolyticus, Streptococcus viridans, Streptococcus fecalis).

Lactic acid:[7] (Streptococcus lacticus).

Spore-forming aerobes: (B. anthracis,[8] B. subtilis[9]).

Thermophilic:[10] (T-60, Illinois Collection).

With reference to the foregoing tabulation two points may be considered before proceeding further. They deal with the situation in the anthrax bacillus and in B. pyocyaneus. For neither of these organisms does d'Herelle thus far (1926) admit the discovery of a bacteriophage, as seems to be implied by their omission in his summarizing table (p. 269),[248] although a lytic principle for the former species had been mentioned by Pico[390] in 1922, and a lytic agent for the latter species had been described by Canzik[89] and several others, including myself,[224] in 1923. Pico's lytic agent perhaps accomplishes little more in the way of lysis of the anthrax culture than the organisms can often accomplish for themselves, as seems to be indicated by the work of Preisz[393] and the more recent studies of Pesch and Katzu. Similarly, the lytic agent in pyocyaneus manifestly effects no more in the way of erosive reactions (autolysis) than these organisms can accomplish spontaneously when surrounded by suitable growth conditions. I have attempted several times to obtain from sewage (from which I have seldom failed to isolate a bacteriophage for many other species) a lytic agent for B. anthracis and for B. pyocyaneus. With the former I have in no case succeeded; while with respect to the latter I have readily secured

[6] A bacteriophage active against streptococcus was reported by Piorkowski[391] in 1922; also by both MacKinley[323] and Clark.[101] In the last two cases, however, the data as yet published have not served to make clear the actual facts involved. Most recently, Dutton[141] has reported a streptococcus bacteriophage. From the data presented, however, the lytic action seems to be of a low order and does not correspond with the principles for Shiga, coli, etc. Evidence is present that the dissociation phenomenon accompanied the reaction. With the assistance of Eugenia Dabney and E. M. Brill, I[228] have obtained from sewage a lytic principle of the typical sort active against Streptococcus fecalis, and Faith Hadley[499] has separated the S and R forms of this organism in colony and in culture. The relation of Streptococcus fecalis to the more typical streptococci (hemolytic and greening), however, is not perhaps clearly established (Hadley and Dabney[228]). Beckerisch and Hauduroy[44] have reported a lytic agent for the "enterococcus," which many investigators regard as identical with Streptococcus fecalis.

[7] I have observed the dissociation of Streptococcus lacticus, which was also probably observed by Joseph Lister[306] as early as 1873, and Eugenia Dabney, a student in my laboratory, has succeeded (1926) in isolating a lytic principle for this organism (Hadley and Dabney[228]).

[8] Many older records indicate the dissociation of the anthrax bacillus. The phenomenon has, however, been clearly verified in our laboratory by W. Nungester,[499] with complete recognition of the two fundamental culture and colony types and probably several others besides the S and R forms. A lytic principle has been reported by Pico[390] in 1922, although this circumstance is not recognized by d'Herelle[248] (1926).

[9] M. Soule[450] was the first clearly to demonstrate the dissociation of the hay bacillus (subtilis) and to show the characteristic S and R colony and culture types. A bacteriophage for B. subtilis has been reported by d'Herelle.[246]

[10] Dr. Stewart Koser,[285] while working in my laboratory during the summer of 1926, succeeded in isolating from sewage a bacteriophage active against a true thermophilic microorganism (growing well at temperatures of 60 C. or above), and effective in producing typical lysis and inhibition in broth or on agar slants at a temperature of 59 C. within the usual time limits. He also succeeded in obtaining the dissociation of this culture. The exact bacterial species concerned is not known, but passes under the symbol of "T-60" of the University of Illinois collections.

a filtrable principle which produces in fresh, sensitive cultures much the same type of erosive action that is commonly observed to occur spontaneously in sensitive laboratory strains. In view of these circumstances, it seems to me strongly suggested that the erosive phenomena occurring in B. anthracis and in B. pyocyaneus cultures, but not in other bacterial cultures (except in the case of Monilia perhaps, as recorded by Sonnenschien [448]), so far as I am aware, represent a reaction analogous to bacteriophage lysis; and that this is the only form in which bacteriophage can manifest itself in these bacterial species. I have repeatedly insisted [224, 226] that the phenomenon of transmissible bacterial autolysis must be conceived as sufficiently broad in its power of manifestation to include these "aberrant" cases, which d'Herelle [248] has unfortunately relegated to the field of "bacterioclysis," a "bacterial disease," without foundation in fact or theory, but highly useful to the proponents of the virus theory of the bacteriophage as a convenient repository for illegitimate and otherwise troublesome phenomena.

Whatever other common factor may support a similarity between active microbic dissociation and transmissible autolysis, as exemplified particularly in the cases mentioned above, there thus exists one such factor in their mutual relation to the phenomenon of microbic instability, and the general trend of the dissociative reaction. In the operation of both there is significantly concerned the disappearance of a relatively unstable type of organism (S), together with the birth of new, different, and usually more resistant types (R), usually characterized by foreshortened coccobacilli or coccus forms. In both, moreover, the unstable form disappears by a process of lysis or of transformation. In active dissociation this lytic action may be so slow as to be almost inappreciable, or it may be fairly rapid. It may extend through the "lifetime" of a single culture and continue in that succeeding (lytic type), or it may culminate within a few days or weeks. It involves the complete or partial disappearance of the "normal" or S type of culture, either over broad areas or in isolated spots on solid cultures, and usually leaves behind a residue whose nature and appearance varies with the intensity of the dissociative process and which contains the modified, resistant forms. In transmissible autolysis (passive dissociation) on the other hand, the disappearance by lysis is sudden; it may cover a day or two or it may culminate in a few hours. Such an autolysis likewise involves the actual or apparent destruction—at least the disappearance—of organisms of the S type, and leaves behind a population of modified, often filtrable and usually resistant, secondary forms which, for a time at

least, may undergo no further change. These similarities are sufficiently striking to anyone who makes a detailed comparative study of the fundamental reactions of the two phenomena.

Relation Between the R Types From Active and Passive Dissociation.—But the similarity may be traced further to the relations between the respective secondary or resistant cultures, already referred to as the primary (R), and secondary (SR, RR) dissociates, a point which has been called attention to by some interesting considerations of Eastwood. The most significant points of comparison involve the following: increased resistance to certain unfavorable conditions of environment and to the lytic principle, together with greater viability and increased susceptibility to phagocytosis (Bordet and Sleeswyk,[64] de Kruif,[121] Griffith,[215] Reimann,[405] Amoss [8] and others); agglutinative or sedimentary form of growth in broth together with instability in the usual concentrations of salt solution; modified antigenic and serologic qualities; and diminished virulence while sometimes still maintaining antigenic protective power. We have already seen that the R type of culture usually shows these characteristics in contrast to the S form which is sensitive, grows homogeneously in broth, retains its original antigenic power and serologic attributes and, if pathogenic, is usually more virulent. We have also seen that somewhat similar changes occur in the transition from the S to the SR type arising after lysis, and probably also in the double transformation from S to RR. In these cases, however, whether we focus attention on dissociation or on autolysis, we observe that the original culture was the same at the start. We also see that the products of change bear close resemblance to each other in many important respects. Indeed, the chief differences between the two processes lie in the following points: the speed of the reaction, and the degree of its transmissibility in vitro.

One exception that might be taken to the foregoing statements regarding the analogies between the R and the SR types relates to their virulence. In this respect d'Herelle [248] has pointed out that cultures resistant to lytic action are more virulent and less phagocytable than their respective normal sensitive progenitors. On the other hand, it has been amply demonstrated in the preceding pages that the R cultures from normal dissociation are both less virulent and more phagocytable than the original S cultures—at least in many pathogenic species. In order to support his view d'Herelle mentions several instances. Bordet and Ciuca [63] for example reported that a resistant coli culture was less

phagocytable and more virulent for laboratory animals than the normal culture. Similar observations were made by Gratia [211] for B. coli. Davison [116] noted a similar phenomenon in B. dysenteriae Shiga, and d'Herelle [248] for B. pestis. In the last instance 0.000.2 cc. of the SR culture killed guinea-pigs in 46 to 50 hours while this result was obtained only by the injection of 0.1 cc. of the normal culture (S). D'Herelle stated, however, that among the secondary colonies there occurred great variation with respect to virulence; and that associated with colonies of increased virulence, he found others completely avirulent. These differences, according to d'Herelle, were due to various "mutations" produced by the bacteriophage. "These mutations do not involve all the characters of the bacterium, but only a certain number of them, varying from a single bacterium to another, even in a single culture. With one bacterium the character of 'agglutinability' will be modified, with another, the character of 'virulence,' and with a third both of these will be changed at the same time. All that may be predicted is that, usually, there is a reduction in agglutinability and an increase in virulence" (p. 219).[248] Thus it appears that, among the organisms resistant to lytic action, d'Herelle admits that nonvirulent forms do appear; and the question at issue therefore resolves itself largely into a matter of the predominance of these or of the virulent form. The answer will need to await further study. It may be noted, however, that Fejgin [168] has presented evidence opposed to d'Herelle's conception. She was able to demonstrate that her Shiga secondary cultures that were resistant to lysis not only lacked virulence, but also showed no power of toxin production. Clearer evidence opposed to d'Herelle's view is found in certain experiments performed by Bronfenbrenner and Korb,[73] having this point particularly in mind. These investigators had already obtained a bacteriophage for B. pestis caviae (not one of the Pasteurella group) and ascertained that lytic principle therapy did not influence the course of experimental mouse typhoid produced with this organism. In studying further the reason for these negative results they had occasion to compare the virulence of the resistant, secondary strain with that of the original culture. To state the results briefly, the cultures that were resistant to lysis were also quite lacking in virulence. Neither through animal inoculation, nor by passage in vitro, did these resistant strains easily recover their susceptibility or their virulence. In this respect, however, there appeared to be two groups of resistants. In broth culture one group gave an agglutinative form of growth; and these organisms reverted to suscepti-

bility and virulence after five to seven daily transfers. The second
group in broth gave homogeneous clouding; and these failed to become
susceptible (or virulent) even after 120 daily passages. Both groups
were stable after 25 passages on agar. In analyzing these results the
authors pointed out (also in opposition to the view of d'Herelle) that
the production of resistant strains is the result of a selection of variants
already existing in the parent culture. This conception was also voiced
by Gratia; and, it cannot be doubted, represents the situation that
actually exists with relation to the generation of the SR forms of
culture. Similar results indicating the nonvirulence of the lytic-resistant
cultures of B. pestis caviae were published subsequently by Bronfen-
brenner, Muckenfuss and Korb.[500]

In summarizing these somewhat conflicting statements regarding
the virulence of these cultures that are resistant to the bacteriophagic
influence, it must be said that the issue remains open. Although such
an increased virulence of the resistant types as d'Herelle pictures in his
latest book is the outcome naturally expected in accordance with
d'Herelle's theory of the bacteriophage, its truth is not supported by
sufficient evidence to permit its acceptance at the present time. Indeed,
I believe it might be stated without exaggeration that present evidence
favors the opposite view; namely, that the cultures resistant to lytic
action are commonly less virulent than the normal culture type, and in
this respect correspond with the majority of the R forms arising through
the course of normal active dissociation. Moreover, I have little doubt
that, when the problem has been studied with sufficient care, and in a
sufficiently large number of of bacterial species, it will be found that the
partly stabilized secondaries to bacteriophage action possess some
residual virulence, as also is the case with the partly stabilized secondaries
from active dissociation; but that the fully stabilized secondaries arising
under the lytic stimulus are commonly nonvirulent, corresponding per-
fectly in this respect with the socalled "extreme" R types from microbic
dissociation, so far as these have been studied up to the present time. To
the above it may be added that, with reference to lytic principle prophy-
laxis and therapeutics, this situation is the most to be desired. If the
power of the bacteriophage is not sufficient to destroy all of the virulent
S forms, it is of advantage that the remaining organisms should be
modified into a nonvirulent, rather than into a more virulent, culture type.
We should, however, perhaps hold ourselves open to the view that the
relation between virulence and the form of culture resistant to lytic
action (SR) may eventually be found to vary with the bacterial species
concerned.

Similarity with Reference to Serologic Reactions.—Among the changes revealed by a bacterial culture in the state of "active or latent resistance" to the bacteriophage, d'Herelle [244] has mentioned as most common the loss of agglutinability with specific antiserums. Also in 1926 he states: "The loss of agglutinability seems to be related to the degree of acquired resistance, for the refractory state is accompanied by a complete inagglutinability and there is only a diminution if the resistance is partial" (p. 218).[248] D'Herelle cites as instances of this fact observations on B. coli, the dysentery bacteria, B. typhosus, B. gallinarum and B. pestis. Gratia [211] has given a similar instance for B. coli strains possessing different degrees of resistance to the bacteriophage. D'Herelle [248] has further pointed out that typhoid bacteria when first isolated from the patient are not only inagglutinable but also resistant to the lytic principle; also that, after agglutinability has been restored by some passages on artificial culture mediums, the organisms become susceptible to bacteriophage action. He (p. 254)[248] has also mentioned obtaining from cultures of B. paratyphosus B mucoid colonies which were resistant to lytic action. These were undoubtedly the mucoid transitional (O) often described in the literature.

From the instances mentioned above, and many similar experimental data, one may certainly conclude that the bacteriophagic reaction is such as to generate cultures which possess marked inagglutinability, and at the same time greater or less resistence to the bacteriophage. But we have also observed in earlier pages of this work that these are exactly the characteristics of cultures of the O or R type which have been derived as dissociates from normal, sensitive culture; and that similar R type strains can be obtained at any time as the direct result of either "spontaneous" or of "forced" dissociation. In other words, the bacteriophage, at least as interpreted by d'Herelle, possesses no exclusive influence in the creation of such culture types. Their appearance, in the culture tube or in the body of the animal, has no other significance than to indicate to us that microbic dissociation has occurred. The force that determines these reactions is intrinsic in the cyclogeny of the species concerned; and their expression is often facilitated, but not exclusively determined, by that agent which we term the bacteriophage

One point of similarity between microbic dissociation and transmissible autolysis relating to serologic aspects of the R types of culture not yet considered is the following. In the section on the relation of microbic dissociation to serologic reactions, attention has been called to the phenomenon of bacterial convergence and to what Schütze has

termed the "serological cosmopolitanism" of the R types. It is now
important to note that the reactions which bring about these results are
by no means limited to normal, spontaneous dissociation, but are observed
equally in the dissociations produced by the lytic principle. The follow-
ing case will suffice to illustrate the point. So-called mutations of
B. paratyphosus A under the influence of the bacteriophage have been
described by Bachmann and de la Barrera.[26] They mention considerable
variation in colony morphology, and some of these forms were undoubt-
edly the resistant type. Cultures made from some of these colonies
revealed marked agglutination in antityphoid serum. With further
transfers of the mutant line this was increased to 6,000 while there was
no increase of agglutinability in the original paratyphoid A serum
(1600). In addition, an antiserum was prepared against the fourth
subculture generation of the "mutant." This serum agglutinated the
homologous (mutant) culture at 3,200, B. typhosus at 800 and the
original paratyphoid at 800. A serum prepared against the sixth sub-
culture generation of the "mutant" agglutinated this culture, also B.
typhosus, but quite failed to agglutinate the original paratyphoid culture.
These results were upheld by absorption tests. Commenting on these
findings, d'Herelle [248] states, "These facts can only mean that there
had been a transformation of the antigenic properties brought about
through the action of the bacteriophage." D'Herelle also calls attention
to Baerthlein's somewhat similar case of alleged transformation of
B. paratyphosus B into B. typhosus and wonders whether the bacterio-
phage may not have been present in this "mutation." With reference
to B. coli and its noteworthy "mutations" he mentions the fact that
symbiosis with the bacteriophage in the intestinal tract is the normal
state of existence for B. coli, and suggests it is for this reason that coli
is a "mutant species." From these several observations, unless one
wishes to assume that the bacteriophage is present in all dissociative
reactions, and is the determining factor in the "mutations" that accom-
pany them, one must believe that, for some reason, the changes which a
culture undergoes in the course of dissociation occurring "spontaneously"
are duplicated in nearly every point by the changes which a culture
undergoes when submitted to the influence of the lytic principle. This
result can leave us only with the thought that the two phenomena are
closely related in effect; and, in all probability, in respect to cause.

*Similarity with Reference to the Generation of Filtrable Forms of
Bacteria.*—The comparison of the secondary cultures arising from these

two reactions may be carried a step further to deal with an aspect of the subject that is of considerable present interest and significance; namely, the granular, and sometimes filtrable, stage of the dissociates or related forms.

As in the case of the Much granules in the tubercle bacillus, the Neisser granules in B. diphtheriae and B. malleus, the Babes-Ernst corpuscles in the typhoid bacillus, the granular bodies observed in spirochetes (Wolbach [489]), and others of an apparently similar sort, most granules of bacteria and most "globoid" or "granular stages" (so-called) of bacteria, have been regarded with suspicion so far as representing viable forms, or playing a rôle in the further life-history of the culture, is concerned. The same attitude toward "involution forms," as maintained by Loeffler, and by Fischer [176] many years ago, remained quite unquestioned up to, and well beyond, the work of Hort [254] a decade later. The severely critical attitude toward such forms, which has descended to bacteriologists along with the mantle of Koch, has doubtless been justified by the circumstance that, although they may be found in quite young cultures, they have often been observed in old or otherwise degenerating cultures, or in organisms submitted to the prejudicial action of normal or immune sera. If any significance at all has been attached to such bodies (particularly small granules) it has usually been in conformity with the teachings of Günther [218] and of Loeffler; namely, that they were "Absterbenerscheinungen," concerned with degeneration changes, and not otherwise of significance. A few investigators (Leishman, Balfour, Hindle and Fantham) have held—for spirochetes—different views, as also did Fontès [182] for the granular bodies which he observed in cultures of the tubercle bacillus in 1910. Of their frequent association with apparent degenerative processes in the cell there can be little doubt, and some of them probably possess no other significance; but it is also becoming clear that some of these "degenerative" changes may, themselves, be of significance as evidence of dissociation in the life of the culture. Moreover, some of these granulation and related changes may occur even in the first hours of growth of quite fresh culture. Indeed, it appears that more than one phenomenon may be hidden beneath Loeffler's "Absterbenerscheinungen." But let us turn to certain facts observable in cultures of several bacterial species.

In active dissociation the reaction is often accompanied or preceded by the generation not only of long filamentous but also of multitudes of minute coccus forms and still smaller, granular, "globoid" or gonidia-like bodies, apparently resembling some of those first described as

gonidia by Conn [103] for Crenothrix. These have been pictured frequently, and especially by Fontès,[182] Almquist,[3] Hort,[254, 257, 258] Löhnis,[310] Mellon [337] and Enderlein.[160] I have observed them often in dissociating cultures of B. coli, B. typhosus, Bact. pneumoniae, B. lepisepticum, B. malleus, B. diphtheriae and in some unidentified organisms from air and water. Faith Hadley [499] has observed them in Streptococcus fecalis. Their mode of origin has been followed with special care by Almquist,[3, 4] and by Enderlein. In cultures of B. malleus and B. diphtheriae these bodies may be detected in normal cultures in small numbers but they are often the preponderant form at certain stages in dissociating cultures. They vary in size from the limit of vision up to a size merging with the distinct coccus and coccoid forms. Unless they are present in large numbers, or are seen in the course of formation, they are often admittedly difficult to differentiate from granular bodies present in known degraded cultures. As I have observed in B. malleus and B. diphtheriae, the transfer to broth from an agar slant culture in the state of active dissociation, and containing many filamentous forms, together with granular bodies and cocci, may result in a few hours' time in the almost complete disappearance of the bacillary and filamentous types and the survival (and apparent active multiplication) of the gonidia-like bodies only. This does not occur in normal cultures similarly treated. These cultures may be stabilized in the coccoid state and apparently remain so for a considerable time. Culture reactions of this sort have been described by Hort,[258] Mellon,[326, 327] Enderlein and others. When transfers of the granular or small coccus stage are made to agar slants growth in the first generations may be absent or it may take the form of an almost invisible growth-film. Microscopic examination of such cultures usually reveals granules or "globoid bodies" mixed with small cocci and occasionally rod forms (Breinl,[70] Fejgin,[169] for B. proteus). I have filtered through Berkefeld N candles such cultures coming from the dissociation of B. diphtheriae, B. malleus and B. coli, and have observed that, after a few days, the filtrates sometimes revealed a faint opalescent growth which showed itself microscopically in fresh preparations as a mixture of minute granules, cocci and small bacillary forms. I have not succeeded as a rule in obtaining such growths in Berkefeld W-filtrates. The demonstration of filtrable forms of bacteria has, however, not been uncommon in recent times, as we have noted in the case of the tubercle bacillus, the bacillus of Johne's disease, B. proteus and other forms; and these results have done much to validate older observations on the "filtrability of bacteria." Some of these include

the filtration of the organism of Schweinepest by Lourens,[313] of the relapsing fever spirochete by Novy and Knapp [374] in 1906, by Nicolle and Blanc,[369] Breinl and Kinghorn,[72] and by Wolbach [489] at a later date; the filtration of microgonidial forms of the tubercle bacillus by Fontès in 1910, the filtration of forms of the typhoid bacillus by Almquist [3] in 1911, the filtration of the meningococcus by Hort [259] in 1915; of Azotobacter by Löhnis; [310] of a diphtheroid culture by Mellon; [325] of Streptothrix by Mellon; [325a] of his "spore-forming coccus" by Pryor; [398] of Streptococcus strains from encephalitis by Evans,[161, 162] confirming earlier reports of the same phenomenon by Rosenow.[412] The work of Fontès, Hort,* Almquist, Mellon and Enderlein is such as to relate the filtrable forms of bacteria to certain developmental stages (microgonidia, gonites) commonly observed in the process of microbic dissociation. In other cases we are not aware of the existence of such a relation from the nature of the data reported. In the tubercle bacillus distinct cultural dissociative changes have not yet been clearly reported, although cell transformations which usually accompany dissociation have been pointed out by Fontès,[182] Enderlein,[160] Karwacki,[507, 508] and perhaps by Vaudremer.[472]

The preceding records of filtrable forms of common bacteria, arising either with or without evidence of dissociation, are perhaps of sufficient significance in themselves; but it is their counterpart in the products of bacteriophagic action that I desire particularly to make clear. And here we shall see that there exists an interesting parallel.

First Hauduroy [234, 235] and later d'Herelle and Hauduroy [249] pointed out the common existence of filtrable forms of B. coli, B. typhosus, B. dysenteriae, Vibrio comma, Micrococcus aureus and Micrococcus albus arising under the influence of a weak lytic principle working on its homologous substratum. Fejgin [171] has also shown the same for B. typhosus. Accompanying the filtrable bodies were observed minute

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* Hort's conception of the nature of the meningococcus and its relation to infection is of special interest. In 1915 he [259] had performed filtration tests which led him to believe that the clinical form of the organism was filtrable. In 1917 he [355] again reviewed the situation and came to the following conclusions, largely supported by single cell observations.

The socalled "giant meningococcus" is not a bacterium but an ascal stage in the life cycle of an organism allied to the ascomycetes. The ascus may vary in size from 0.2 to 5.0 μ. The meningococcus of Weichselbaum is not a bacterium but an ascospore derived from the giant meningococcus by a process of endosporulation. He believes that the filtrable form previously described is a stage in the life cycle of this organism, and suspects that the reason why the true nature of the meningococcus has not been recognized earlier is because of the exclusive use of solid culture mediums for identification purposes; and also because the organisms have not been carefully studied in the living and unstained condition. In respect to the possible establishing of the meningococcus as one of the Ascomycetes, however, it is perhaps permissible to believe that this organism might incorporate into its life cycle some of the reproductive methods of the Ascomycetes without the necessity of becoming a member of this group. However we may come to regard Hort's conclusions as a whole, the data which he presents to support the view of endosporulation are, to say the least, very striking and entirely in line with similar observations on B. typhosus.

granular bodies and small coccus or coccoid forms. D'Herelle and
Hauduroy reported that the granular bodies arising in the filtrates might
or might not grow on ordinary mediums. If they did grow the culture
might perpetuate the granular form, show a reversion to the original
form or grow in a new form such as coccus or coccoid. Such results
were not uniform: they occurred only in a small proportion of the tubes.
They called special attention to the circumstance that, whatever the
morphologic type of the organism first submitted to the action of the
lytic filtrate—spirillum, rod or coccus—the end was the same, a granular
or coccus type. It will be borne in mind that Bail [33] has seen in some
of these filtrable stages a significance ("splitter" theory) uniting them
with the actual agent of bacteriolytic action. According to his view these
particles propagate only when provided with nutrient material from the
living organisms. Otto and Winkler,[380] moreover, have regarded the
bacteriophage as constituted of minute fragments of bacterial substance
possessing the properties of enzymes. Also, Bail, Otto and Munter [379]
and many others have reported obtaining the bacteriophage from filtrates
of old normal cultures.

The recent work of Enderlein [160] has given a view of much interest
regarding the significance of small coccus stages and granular or
"globoid" bodies in cultures of many common bacteria, including Vibrio
comma, B. diphtheriae, B. tuberculosis, B. anthracis, and also the spiro-
chetes; and particularly with reference to the so-called "death" of the
old culture mass. According to Enderlein, there has existed a great mis-
conception as to what really constitutes the death of a culture. Ordi-
narily, when we attempt to subculture from an old agar slant, for
example, to another slanted medium, and observe that no growth results,
we conclude that the old culture is dead. Not necessarily so, according
to Enderlein; and the reason is as follows: In the old culture the
organisms have undergone a transformation. If a rod form, the nor-
mal bacilli or the filaments generate coccus or coccoid bodies (gonidia)
which are still viable and easily cultivable on fresh medium. Later,
however, as the culture ages (and particularly if placed in a window in
diffuse sunlight), the gonidia give rise to smaller, granular elements
(gonites) which cannot be cultivated on a solid medium but which, when
planted in broth, become transformed further into the sex cells
(spermites and oites). In a similar manner, neither of these sexual
forms is independently cultivable; but after fertilization has taken place
(in a liquid medium), as it does about five and one-half to seven
hours after seeding the gonit culture, the newly fecundated oites are

cultivable either in broth or on agar and proceed to generate the normal cell type (mychit), thus completing the developmental cycle or cyclode. The terminology employed by Enderlein in delineating the detailed features of his comparative bacterial cytology is extremely elaborate. The fact remains, however, that whatever we may term these granular bodies and the elements from which they arise, they have been observed repeatedly in many bacterial species and have been traced to the gonidia as a point of origin (Almquist,[3] 1911, for B. typhosus). Enderlein's conception of the actual mechanics of this phenomenon, isolated phases of which have been reported by numerous workers, to say the least, offers the most logical hypothesis that has been made available to explain the alternation of coccus and bacillary phases of growth in the same culture; and for the alternation between filtrable and nonfiltrable stages in the life of the culture, as observed by d'Herelle and Hauduroy and many others. Thus, to conclude the reference to the work of Enderlein, the most common of the "filtrable forms" of bacteria are not "fragments" endowed with "regenerative" power, but definite stages in the cyclogeny of the species, and for the most part the microgonidia (Cohn, 1870) and the gonites (Enderlein, 1925); or in some cases the spermites, according to Enderlein's interpretation.

There is thus seen to be a marked similarity between some of the ultimate products of active and passive dissociation with reference to these granular, secondary types and their capacity for, and manner of, regeneration; and the same is true of the filtrable forms of bacteria. Although, as d'Herelle states, filtrable forms certainly do arise under the influence of the lytic agent, manifestly the lytic agent is not a necessary factor in the process. In this connection it is interesting to note that in earlier days one of d'Herelle's chief objections to regarding the Twort phenomenon as an example of actual bacteriophagic action was that some of the organisms, rather than being wholly destroyed in the lysing, transparent colony, were transformed into minute cocci, still observable in the lysate. Later he points out a similar transformation (in broth) of M. albus and M. aureus as evidence of the modifying influence of specific bacteriophage, together with the ultimate production of "symbiotic types" (filtrable cell fragments parasitized by the bacteriophage). Here we have the remarkable picture of two filtrable organisms living together in a state of symbiosis!

In all these studies dealing with granular and filtrable forms of bacteria we are naturally treading on difficult and uncertain ground. From the results thus far attained, however, I believe we may be

reasonably certain of two things: first, that aside from the lifeless
colloidal fragments which undoubtedly constitute the chief background
in many lysate examinations, there actually exist living, ultramicroscopic
and filtrable forms of bacteria; second, that the generation of these
bacterial "fragments" or invisible corpuscles stands in some relation to
processes involved in microbic dissociation, either active or passive.
Although themselves often filtrable, and capable of generating experi-
mental infections, as now shown by many investigators, the relation of
these bodies to the class of recognized filtrable viruses, as suggested in
the interesting contribution of Ch. Nicolle [368] in 1925, must be left
an open question. In any case, however, we cannot close our eyes to the
possibility that some of the bacterial forms which we obtain for culti-
vation and study may, as Hort has expressed the matter, and as Ender-
lein has attempted to demonstrate, merely be the "vegetative side issue"
of a life story, the nature of which, for the most part, is still beyond our
knowledge.

Comparison with Reference to the Medium.—With further reference
to the similarities between microbic dissociation and transmissible auto-
lysis, there may be noted a relation to certain conditions in the medium
which permit or favor the reaction. It is commonly accepted that trans-
missible autolysis does not occur in a medium which does not permit
growth. The same appears to be true of active dissociation, although it
does occur, indeed is sometimes favored, on mediums much impoverished
in nutrient qualities (Braun and Schaeffer,[66] Feiler,[167] de Kruif [118, 119]).
While dissociations may proceed rapidly in plain broth or peptone water
(de Kruif for Bact. lepisepticum), Griffith has stated, with reference
to pneumococcus dissociation, that the organisms must grow before the
S to R transformaton can occur. Soule [450] also has ascertained quite
recently in his study of dissociation in B. subtilis that, when the S type
is suspended in distilled water or physiologic salt solution and plated
after varying intervals, no dissociation is found to have occurred.
While the microbes live, but do not multiply, they do not dissociate.
Control broth tubes similarly inoculated revealed growth and from 20 to
30% dissociation in a few days time. Quite in line with these results,
I have found that the S forms of B. coli, Bact. pneumoniae, and
B. pyocyaneus, when suspended in salt solution, adhere to type as long
as they live. Although additional tests of this matter in other dissociat-
ing species are needed, all present evidence points to the view that
neither transmissible autolysis nor microbic dissociation can occur in a

medium which does not permit growth of the bacteria concerned. This circumstance might lead us toward the conclusion that both dissociation and transmissible autolysis are functions of growth, as is also intimated by much other evidence.

Again, it is to be noted that a similar reaction of the medium is important for both. The reaction most favorable for autolysis was established by Gratia [209] as about P_H 8.5 for B. coli. D'Herelle,[248] however, has stated most recently that P_H 7.8 is the most favorable reaction for the action of the bacteriophage. The same reaction was found most desirable by Griffith [215] for the dissociation of the pneumococcus; also by Atkin [21, 22] for dissociation of the meningococcus and the gonococcus. De Kruif [119] found an alkaline reaction most favorable for the reaction in Bact. lepisepticum. For his study of dissociation in B. typhosus Hort [258] used an alkaline agar; and the same was true in the case of Eisenberg for various species. Preisz [393] obtained his dissociating anthrax cultures in a slightly alkaline medium. I have been able to obtain "spontaneous dissociation" (accompanied by macroscopic areas of lysis) in more than a dozen cultures belonging to various groups, merely by growing on rich beef-infusion-agar containing proteose peptone and adjusted to a reaction of P_H 7.8. In most cases the dissociation begins after two to four days on the free borders of the growth, thus confirming Hort's [258] observation that in his typhoid cultures the "mutants" were always found most numerous on the free edges. In my experience, I have not been able to obtain dissociations accompanied by macroscopic areas of lysis on mediums of low P_H value, and Atkin showed that P_H 7.4 or 7.5 did not commonly permit the appearance of papillae on his colonies of meningococcus and gonococcus. B. pyocyaneus, as I have ascertained, may be maintained permanently in the lytic and lysogenic state by monthly transfers on mediums of 7.6 or above. If, however, the reaction drops to 7.0 or below, the lytic phenomena rapidly disappear or are represented merely by metallic flecks on the surface of the culture. Although, as d'Herelle [248] has pointed out, the lytic reaction caused by the bacteriophage may be adapted to conditions of acidity, there is universal agreement that alkaline conditions favor in a marked way both microbic dissociation and transmissible autolysis.

Comparison with Reference to Proliferative Growth.—Another observation in which the similarity between the two reactions is shown relates to the phenomenon of proliferative growth. This is manifested

as a rule preceding the critical reaction in transmissible autolysis, and is usually observed in such acute dissociations as reveal themselves by the formation of macroscopic areas of lysis on solid mediums. The highest point of excess growth terminating the proliferation, and just preceding lysis, has been termed by Bordet the "critical stage"; and in a former paper I have termed it the "lytic threshold" (in B. pyocyaneus and other cultures). Attention to the same point in the dissociation of B. anthracis has been called by both Preisz and Wagner. In both instances one may note a degree of increased growth-energy which in many instances is so great as to justify the term, proliferation. It may be borne in mind, however, that in such slow-going dissociations as occur on "starvation agar" or phenol agar (Braun and Schaeffer,[66] Feiler [167]), or in organisms undergoing adaptation to dyes (Revis,[404] Stearn [452]) such exalted growth may not occur. In transmissible autolysis the period of proliferative growth is terminated by the sudden disappearance of the majority of the S organisms present, while in active dissociation it is terminated by a much slower and progressive disintegration and transformation of the S type, some of which may, for a variable time, continue to support a changing bacterial population in flux toward the O or the R form of culture. It seems clear that, in dissociation at least, the proliferative growth marks the development of the intermediate or O culture type.

Comparison with Reference to Lytic and Lysogenic Cultures.—Next, in pursuing our consideration of the similarities existing between active microbic dissociation and transmissible autolysis, it is necessary to mention a matter of considerable theoretic importance relating to the rôle played by a certain type of cell (and sometimes, colony) in the culture in the perpetuation (and perhaps in the initiation) of, not only the simple dissociative, but perhaps also the frankly bacteriophagic, reaction. On an earlier page I have called attention to certain observations of Preisz, Katzu and also Pesch on lysing colonies of the anthrax bacillus; also to the observation of Pesch that certain translucent colonies exert a "repressive influence" on the growth of the whitish, more opaque S type. I have also mentioned the observations of Firtsch and of Bernhardt on the curious reactions of the "third colony intermediate." There also has been pointed out the observation of de Kruif on the repressive action of the R colonies of Bact. lepisepticum on the growth of the S colonies. Also the observations of Ørskov and Larsen on the inhibiting action of filtrates of broth cultures of their curious self-lysing variant (paradysentery) upon the growth of normal culture. Also some obser-

vations of Arkwright and of my own on the same point. I have in addition called attention to the peculiar colonies which I have seen in cultures of B. diphtheriae and staphylococcus, and which I have termed "invisible colonies" because of their ability to grow in the midst of the culture mass in an almost invisible form; and in such a manner as to produce the semblance of lytic areas, together with the subsequent characteristic appearance of secondary colonies. Indeed, anyone working much with dissociating cultures on solid culture mediums cannot fail to be impressed by the inhibiting, and sometimes apparently lytic, effect produced by certain kinds of colonies in the culture mass. Our conclusions from these observations were that, through active dissociation there may arise certain cells that are instrumental in reinitiating, and perhaps in perpetuating, the dissociative reaction. Indeed, Otto, Munter and Winkler, according to d'Herelle,[248] have reported raising the level of such an antagonistic reaction to the grade of an actual transmissible autolysis merely by passing normal Shiga cultures through heated filtrates of homologous broth cultures. I believe that studies in this field are among the most important that can be directed toward the solution of both acute microbic dissociation and transmissible autolysis.

Turning the line of our inquiry now more directly to the problem of transmissible autolysis, we have also seen that the primary rôle of the bacteriophage, apart from its more obvious lytic and inhibitive influence, may be regarded as that of an agent effecting a dissociation of the sensitive culture into various components, among which the O and R types come into special prominence. We recall that, for Bordet, the bacteriophage is a factor in the production of "mutations," and that for Arkwright [17] "it is a substance which disturbs the balance of the growing bacteria in the direction of autolysis." There certainly exists little if any direct evidence that the bacteriophage, itself, is the thing that accomplishes lysis. Among the variants produced by the action of the bacteriophage in a sensitive culture some may still be sensitive (Bordet,[61] Arkwright [17] and others), some may be resistant without "carrying" the lytic agent, while still others are resistant and do "carry" the lytic agent (d'Herelle,[248] Bordet,[60] Gratia,[211] Lisbonne and Carrére [305]). The last are called lysogenic cultures and are able, through contact or through their filtrates, to precipitate lytic or growth-inhibitive reactions in normal, sensitive culture. Furthermore, in so doing, they cause the generation of more organisms like themselves, as well as more sensitives (perhaps) and more nonlysogenic resistants. D'Herelle and Hauduroy [249] have made special provision for these lysogenic forms, either as

organized cells of normal SR type, or as filtrable forms, "existing in symbiosis with the bacteriophage." While this conception of symbiosis between a bacteriophagic virus and filtrable forms of resistant bacteria requires a stretch of imagination for which many bacteriologists may not be qualified, I believe this last point mentioned above is an admission, on the part of d'Herelle, which represents the greatest, recent, single advance toward our understanding of the nature of the bacteriophage and of the probable mechanism of lytic action; and to this point I shall subsequently return.

In summarizing the most essential points of the last two or three paragraphs I would lay stress on the following: In ordinary, active dissociation certain cells may arise which exert a repressive and inhibitory or even lytic influence on the normal, sensitive components of the culture; in transmissible autolysis also, certain cells are found which are themselves resistant to lysis but which, at the same time, are capable of generating lytic reactions in sensitive culture; these lysogenic cells may exist in the common, organized form or in a filtrable form, and sometimes may not show obvious growth on solid or in liquid mediums; it may be quite gratuitous to link into symbiotic union with these cells or cell "fragments" a hypothetical bacteriophagic ultravirus in order to explain their lysogenic potentialities.

Regarding the similarities between dissociation and transmissible autolysis one final point may be considered; namely, the existence of what has been termed "border-line types" of reaction. In most cases it is not difficult, through the examination of a culture, or of its "built-up" filtrates, to reach a conclusion as to its status—whether more closely related to active dissociation or to transmissible autolysis, as these terms have been defined. Such clear differentiation, however, may sometimes prove difficult in such cases as represented by colony lysis in B. anthracis (Preisz,[393] Pesch, Katzu), in B. pyocyaneus (Canzik, myself and others) and in Monilia (Sonnenschien). There are, furthermore, other instances in which a decision is even more difficult; namely, in the "suicide cultures." In all of these, we perhaps observe "border line cases." Among them are some which lean to the side of transmissible autolysis by reason of the fact that they give rise to filtrates which have a sort of lytic action that is easily transmissible in series. Others, however, approximate active dissociation by reason of a more tardy reaction, together with the impossibility of isolating filtrates which possess the outstanding features of the typical bacteriophage. It is somewhere in this series, perhaps, that Twort's case might be placed;

also the case of pyocyaneus lysis. By several, including myself, these have been regarded as examples of actual bacteriophagic action although d'Herelle [248] has excluded both from his category of classical lysis. In his latest contribution he still maintains that there are two "diseased states" in bacteria, indicated by the phenomenon of bacterioclysis, and the phenomenon of the bacteriophage (bacteriolysis). The first, involving merely bacterial "fragmentation," is typified by Twort's [468] case; only the latter, involving complete dissolution, is related to the bacteriophage. Therefore the autolysis of B. pyocyaneus would also be an instance of bacterioclysis according to d'Herelle. The improbability of this view I have already discussed.

In this connection, however, I wish to digress sufficiently to add that further observations which I have made on pyocyaneus have only increased my conviction that the mode of lysis which I described for this organism in 1924, while differing in certain respects (mainly speed of reaction and degree of transmissibility) from the classical autolysis of d'Herelle, belongs to the same category of phenomena. But in pyocyaneus the lysis is both active and transmissible; it is of the borderline type. The fact that it is spontaneous under certain cultural conditions has been impressed upon me especially by the circumstance that, of seven typical pyocyaneus cultures received from other laboratories (where lytic manifestations had never been observed) six became lytic within a short time after transfer to the rather alkaline beef-infusion-agar commonly used in my laboratory for all dissociation work. From several of these cultures a lytic filtrate has been obtained which in recent tests has sometimes accelerated the autolysis of the lysogenic culture itself, and in addition has precipitated a fresh, sensitive culture into similar lytic manifestations. And most recently I have been able to show that there exists in sewage-polluted river water an agent which is able to induce in other sensitive pyocyaneus cultures, as well as in many other bacterial species, the same sort of dissociative change. I therefore regard the case of autolysis in pyocyaneus as one which stands near the borderline of reaction, and one which is interpretable under the heading either of transmissible autolysis or active dissociation. Of two points there can be no doubt: that it is often spontaneous; and that it is weakly transmissible. It is for this reason that I have spoken of this organism as being both lytic and lysogenic.

To the foregoing considerations I would also add one further point regarding the relation of the agent of transmissible autolysis to the culture undergoing dissolution, an aspect of the matter which has been

mentioned on a previous page. In all cases thus far reported in the literature on the bacteriophage (d'Herelle [248]) the lytic principle has been found to operate only at a temperature which is close to, or below, the maximum growth temperature of the common bacterial species. Thus, in certain especially heat-resistant strains of B. coli d'Herelle has reported lytic action at 45 C. The question therefore arises, what would be the situation with respect to a lytic principle operative on a strictly thermophilic organism growing at 60 C. or perhaps higher, but only slightly at 37 C., and not appreciably at lower temperatures? Dr. Stewart Koser,[285] working quite recently in my laboratory, has studied this problem. He has obtained from sewage contaminated water a lytic agent highly active on a still unknown organism of the thermophilic group ("T-60" of the University of Illinois collections) which grows luxuriantly at 60 C. or higher, but less vigorously at 37 C. The lytic principle obtained operates well at 37 C. but much more effectively at 56 to 58 C., and possibly higher, since this aspect of the study has only been begun by Dr. Koser. It is transmissible indefinitely in series and gives small lytic areas at 37 C., but much larger areas (3 to 6 mm.) at 59 C. Broth cultures are promptly lysed overnight at 56 to 58 C., and perhaps to a lesser degree at 37 C.

The complete study of this interesting and, I believe, unique example of bacteriophage activity will be published by Dr. Koser at a later date. For present consideration it is however important to note that, in this case, the thermal requirements of the lytic agent are distinctly correlated with the thermal requirements of the organism of the substratum. Moreover, in the production of this bacteriophage there was no occasion for the slow "adaptation" of a "low-temperature" bacteriophage present in sewage to the thermophilic culture. The bacteriophage revealed itself at once and directly. Shall we conclude from these facts that there exists in sewage a special sort of bacteriophage in the form of an ultravirus, especially qualified to deal with its thermophilic host? Or shall we accept these data as suggesting the view that each strain of bacteriophage stands in some sort of a close biologic relationship to the culture of the substratum, and is perhaps a specialized form of this culture, partaking equally of certain of its biochemical and biophysical growth-characteristics? As Koser [285] has well pointed out, if we continue to assume that the bacteriophage is of the nature of a filtrable virus, foreign to the cell, then we are forced to postulate the existence of a thermophilic ultravirus.

Summary of Section.—In summarizing this section it may be said that there exists a considerable body of scattered evidence revealing a distinct similarity between active microbic dissociation and transmissible bacterial autolysis with respect to the nature of the substratum, and the mechanism and results of the reaction. In detail these similarities appertain to the following points.

In both phenomena the reaction involves the disappearance by transformation or by lysis of all, or of a part, of the "normal" organisms. Those that remain are likely to be of modified type (R or SR).

Transmissible autolysis has been found to occur only in those bacterial species which have also been observed to manifest outstanding symptoms of microbic dissociation.

Both the reactions start at the same end of the dissociative scale—that is, with cultures of the sensitive or S type. The secondary or resistant forms of both, moreover, are much more stable, but a "reversion" is possible in both.

In both phenomena the degree and the speed of the reaction, as well as the physiologic state of the product, depend on the degree of concentration or the intensity of action of the inciting stimulus—whether lytic filtrate, antiseptic, immune serum, or some unfavorable condition of environment. In both, "intermediate" cultures are observed.

There exists a striking similarity between the intermediate and the end products in the two reactions, respectively. Among the intermediates are mucoid forms, while the R and SR forms appear as cocco-bacilli in the majority of instances.

The R and SR types differ antigenically and biochemically from the mother culture; and these departures in both, so far as studied, seem to be of the same order, even with respect to bacterial convergence.

The R (from active microbic dissociation) and the SR (from transmissible autolysis) types of pathogenic cultures agree further in that they are commonly nonmotile; also often nonvirulent, or less virulent than the original S form.

The influence of filtrates of dissociated cultures on the S type is sometimes such as to parallel, within limits, some of the effects of the lytic principle on its homologous substratum; and this relates to inhibition of growth and a low grade of transmissibility.

In many instances the conditions of the medium which favor dissociation are the same as those which favor transmissibile autolysis; and this is seen particularly in the reaction, which, for best results, should be alkaline (about P_H 7.8).

Neither microbic dissociation nor transmissible autolysis appear to occur in a medium in which the organisms concerned cannot grow.

In both phenomena it is observed that a heightened growth activity or "proliferation" in cultures is present just preceding the acquirement of the lytic threshold or "critical stage."

In both cases the culture changes involve the generation of "filtrable forms" of bacteria which may or may not grow in artificial culture mediums, and which may sometimes regenerate either the original form or a modified form of culture.

Conclusion: A New Theory of Transmissible Autolysis.—The final conclusions and inferences that may be drawn from the observations reported are mainly reserved for the concluding section of this work.

I wish to say at this point, however, that I believe that the apparently
intimate relation existing between many aspects of microbic dissociation
and transmissible autolysis justifies the formulation of a new hypothesis
of the bacteriophagic phenomenon (especially with reference to the
mode of formation of lytic areas) quite different from that of either
d'Herelle or Bordet, but somewhat more in harmony with the view of
the latter. D'Herelle as we know believes that the lytic area is a colony
of bacteriophage (ultravirus), produced from the growth of a single
"corpuscle" on its proper bacterial substratum (parasitized culture).
Bordet, on the other hand, holds the opinion that the lytic area is the
result of the action of a nonliving growth product (chemical substance)
on a certain type of bacterial cell possessing a sort of hypersensitiveness;
and from whose growth, but subsequent destruction by lysis, is set free
more of the same "substance" which, in turn, effects the dissolution of
neighboring, but previously more resistant, cells. The destruction of
these cells en masse in localized areas produces the areas of lysis. As
will be seen below, my own view, an alternate to both of those men-
tioned above, shares nothing in common with d'Herelle's but depends
equally with Bordet's upon the existence of certain cells of unusual
"sensitiveness." In my case, however, I believe that I have adduced a
certain degree of evidence for the identity of these "sensitive" cells with
some of those belonging to what I have termed the "third colony inter-
mediate" occurring in microbic dissociation. Beyond this, my theory
calls to its support, not the participation of "chemical substances" as
the direct cause of lysis, but of certain biologic elements operative in
some suspected, though still unproved, phases of bacterial reproduction,
which have recently been introduced into the science of bacteriology
through the cytological studies of Enderlein. My own theory of the
reaction may therefore be stated roughly as follows: I make use of
the lytic areas in illustration, because I believe that, if these can be
explained, the explanation of related phenomena will easily follow.

Lytic areas are in reality "vanishing" secondary colonies arising, and
as quickly disappearing, in the mother culture. They are formed by
the same organisms as those of the culture of the substratum, but by
cells *in a different cyclogenic state*, or stage of development, from others
in the same substratum. Having multiplied to a slight extent on the
surface of the medium, they quickly disappear, leaving an apparently
bare area in which there may develop, after a time, the sprinkling of
colonies which d'Herelle, as also Bordet, regards as the resistant secon-
daries, but which are in reality *tertiary* colonies. In other words, a lytic

area represents the site of disappearance of a certain type of inter-
mediate colony which is both lytic and lysogenic: lytic, because it has
the power to undergo a transformation characterized by lysis; lysogenic,
because it can impart the lytic stimulus to certain neighboring cells. But
why, then, do not these lytic colonies arise more commonly in normal,
sensitive culture without the instigation of the lytic filtrate (bacterio-
phage suspension)? This brings us to the second aspect of the theory.

Although the secondary, lytic colonies arise through the multipli-
cation of certain sensitive cells of the substratum, a stimulus to growth
is required. What is the nature of this stimulus? It is the bacterio-
phage—a filtrable stage in the cyclogeny of the same bacterial species
(or of a closely related species); and it accomplishes this end—not
directly by growth and multiplication itself (for, alone, it is nonpropa-
gating), but indirectly by serving as some sort of stimulus—perhaps
fructifying—to the rapid development of certain, young specialized cells
present in the substratum, as mentioned above. These cells react first
by rapid multiplication (proliferative stage), thus attaining the lytic
threshold; then immediately by lysis, accompanied, however, by the
generation of a fresh brood of filtrable forms. These, in turn, serve as
the exciting stimulus to a similar reaction on the part of other sufficiently
young, specialized cells. It is conceivable that the same mechanism
might operate as well in liquid mediums as on solid.

Without attempting to force this theory too far at the present
moment, certain eventualities may perhaps be anticipated. First, how
can such a theory explain the clear relation always shown between the
number of lytic areas produced and the amount or concentration of the
bacteriophagic suspension employed? It may be assumed this is because
the number of cells in the substratum susceptible of receiving the stimu-
lus of the "bacteriophagic corpuscle" is, in young, sensitive (S) cultures
always sufficiently great. The number of the filtrable "units" establish-
ing a "contact" will thus determine the number of sensitive cells that
are to receive the stimulus. Only the cells receiving this stimulus will
develop in such a manner as to form the colonies endowed with the
"vanishing" (i. e., lytic) characteristic. If they do not receive the
stimulus, they may still develop, but perhaps along another path in
the cyclogeny of the species.

Again, one may ask: Why should the reaction (i. e., the formation of
lytic areas, lysis in broth, etc.) manifest itself most definitely in young
cultures? It might be because only the youngest cells are susceptible of

receiving, or being influenced by, the stimulus derivable from the filtrable bodies. If too old, their course of development is perhaps turned into another line.

Further, why are the R type cultures (from microbic dissociation) less susceptible to lytic action, as shown by the reduced number of lytic areas, their smaller size, and the failure of complete lysis in liquid culture mediums; and why are certain SR type cultures (arising from bacteriophage action) refractory to lysis? It seems probable that this is because the type R culture has entered a cyclogenic stage which is nearly destitute of those specialized, sensitive cells capable of receiving the stimulus from the filtrable forms; and because the SR (or, as I have previously termed it, the SR^n) type culture is entirely destitute of those same sensitive cells whose further development (in lysis) depends on the stimulus derived from the filtrable bodies; and, it may be hazarded, upon whose further development, at least in a certain direction, the generation of additional filtrable bodies themselves, depends.

Again, one may inquire, why, if the filtrable form of the organism serving as the stimulus to development of the sensitive (mother?) cells is a normal constituent of bacterial cultures, is not the lytic phenomenon manifested commonly in "normal" cultures? In answer to this I would say that there can be little doubt that the reaction, in a much reduced degree, is often occurring in such cultures; and that it has at times been raised to the level of the classical bacteriophagic phenomenon. Such instances have often been mentioned in the literature and with increasing frequency; but d'Herelle has disposed of them all as cases in which the cultures concerned were "contaminated" with the lytic agent; in other words these investigators have, according to d'Herelle, worked with "mixed cultures." Such rebuttal is incongruous but apparently effectual. Wherever lytic phenomena possessing transmissible characteristics appear spontaneously, there stands the bacteriophage; and, if not explainable otherwise, as a "contaminant."

Finally, the question may arise: If the thing we term the bacteriophage is a filtrable form of the same bacterial culture as that of the substratum, how is it possible that a bacteriophage suspension of B. dysenteriae Shiga, for example, is able to produce lytic areas in a substratum culture of B. coli, or perhaps of B. typhosus? It may be freely admitted that this at present constitutes something of a difficulty. Pending the acquisition of further data, however, we can perhaps regard the matter in the following light.

The sensitive cells of the substratum culture may be stimulated not only by filtrable bodies of the homologous bacterial strain or species, but also by filtrable bodies arising from fairly closely related heterologous species. A diversity of biologic relationship is possible, but it must not be too far distant. Evidence of bacterial antigenic relationships that might suggest such a possibility has been presented in the preceding pages, and concerns the phenomenon that we are coming to know as bacterial convergence, particularly with reference to the R types. We have seen that the R forms arising from microbic dissociation in a variety of organisms, often manifest distinct antigenic relationships, which are not so commonly, if at all, revealed by the corresponding S forms of culture. This community of antigenic structure, as indicated by serologic tests, has been referred to by Schütze as the "serological cosmopolitanism" of the R type. But we have also noted from the observations of Bachmann and de la Barrera [26] that a partially analogous phenomenon occurs with respect to certain SR cultures arising from bacteriophagic action. Considering this apparently close antigenic relationship between the resistant forms of bacteria belonging to heterologous, but somewhat related, species it might seem possible that any stimulating influence (perhaps fructifying) exerted through the filtrable bodies of the homologous culture, could be shared with analogous, filtrable forms from closely related but heterologous bacterial species. If such a circumstance as this were possible, one might anticipate that there would be some correspondence between the specific limits of antigenic relationship, as manifested by serologic tests, and the specific limits of bacteriophagic heterogeneity of action, as manifested by cross tests involving lysis and inhibition. In the data presented in the foregoing pages relating to the serologic aspects of bacterial convergence, there is certainly inherent the suggestion that the serologic relationships of the R forms of heterologous species are roughly characterized by about the same latitude of action that is also observed in the heterologous reactions creditable to the bacteriophage. To ascertain in detail the actual limits of this possible parallel is a highly important matter.

In indulging in considerations of this sort, one unfortunately feels himself drawn further and further from the beaten paths of current bacteriologic thought; and sees here and there the faint shadows of possibilities which, on more sober reflection, he is likely to believe can have no basis in fact. So it is perhaps with the last mentioned aspect of the present theory of bacteriophagic action. One has glimpses of

things possible but still unrecognized in the bacterial world—perhaps hybridization of a sort among bacterial species—who can tell?

Although such a theory of bacteriophagic action as I have presented above finds some support, so far as its major premises are concerned, in many of the facts demonstrated in microbic dissociation, other aspects can be sketched only roughly; and it is scarcely feasible, in our present lack of knowledge, to pursue the conception in further detail at the present time. Whatever its inadequacies may be, I believe it is no more "speculative" than any other theory of the bacteriophage that has thus far been advanced, although d'Herelle's parasitic virus hypothesis is perhaps the more obvious and easier to believe; moreover, it is far simpler in its mechanism. The theory which I have outlined may, however, possess the advantage of affording a somewhat new viewpoint for future experimental work, in which we might regard the bacteriophage "unit" or "corpuscle," not as a foreign filtrable virus parasitizing the bacteria, nor as a chemical substance capable of regeneration by the organisms which it affects, but in the light of a functionally incomplete and ordinarily non-selfpropagating stage (perhaps the "spermit" of Enderlein) in the cyclogeny of the bacterial species. It is obvious, however, that such a conception as I have presented is dependent on the existence of a definite stage, characterized by sexual reproduction, in the cyclogeny of all bacteria manifesting the phenomenon of transmissible autolysis; and even this circumstance is not yet proved. For this reason I anticipate that the theory will have few adherents. However this may be, I may say in concluding this phase of the subject that the views expressed above serve, in part, as a basis for the opinion I have presented elsewhere in this work—that the whole problem of the bacteriophage may ultimately come to appear to us merely as a side issue of the vastly greater and more significant problem of microbic dissociation, in which it will sometime find its place.*

15. RELATION OF MICROBIC DISSOCIATION TO THE PROBLEM OF BACTERIAL MUTATION

Regarding the furtherance of problems of adequate bacterial classification it is easy to observe in the literature of the past thirty years that the most common stimulus activating bacteriologists has been the desire to perfect a scheme of classification rather than to study the causes and

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* Some of the statements and conclusions presented in the latter part of the present section are so closely related to conceptions developed in section 16, dealing with the biologic significance of dissociation, that these two portions of the work should be considered together. At least, the reading of section 16 will make clearer certain aspects of the foregoing treatment of the subject of transmissible autolysis.

limits of variation among bacteria themselves. Much has been written about simple variations, impressed variations and mutations; but, with the possible exception of such works as those of Buchanan and Traux,[78] Wolff,[490] the Winslows [486] and a few others, little has been of value and the majority positively misleading from the viewpoint of genetics. Cole,[104] himself a geneticist and not a bacteriologist, was one of the first (with Wright) to point out the inadequacy of the biological viewpoint of most bacteriologists in these matters; and to draw attention to what he then regarded as the probable nature of the commonly observed changes in bacterial types, as well as to the significance of Johanessen's pure-line concept for bacteria as a group. While it can scarcely be doubted that this concept is applicable to bacteria, that it operates in the manner assumed by Cole ten years ago is unlikely, as he himself would now undoubtedly be the first to admit. It is perhaps true that, of all the variations shown by different strains, many are due to the isolation of biotypes, each possessing its own range of variation, and probably overlapping other ranges.

But Cole assumed further, as many bacteriologists have also done, that if in any pure line originating from a single cell we can detect an hereditary departure from the mode of the biotype, it must be conceded that we are dealing with a mutation in the original meaning of the term. This conception I now believe to be inadequate. Such variations (mutations) Cole believed to be rare among bacteria, at least in proportion to the number of variants properly attributable to the isolation of biotypes from cultures representing a heterogeneous mixture of pure lines. He pointed out, however, a number of instances which he admitted to the mutant group; and he was ready to admit others but for the circumstance that their isolation had not involved single cell methods of cultivation. Since the time of Cole's publication many isolations of "mutants" have been made under conditions which perhaps guarantee variation from a pure line; and which, since they often seem to breed true to the new type, would naturally be regarded as mutants in the usual sense. Indeed the belief in the frequency of bacterial mutation has grown remarkably within the past fifteen years, during which time it has been a common view that the "mutants" have arisen in the progress of "normal reproduction"—meaning, of course, simple fission. In the analyses of the nature and stability of these new accessions to the already burdened archives of bacteriology, some have been reported as returning, sooner or later, to the original type; while others, it has been insisted with equal vehemence, have remained true to the new char-

acters (section 12). This aspect of stability, it may be noted, has done
much to support and even to increase the scientific zeal of the mutation
discoverers. Indeed the frequency of alleged mutations has been so
reflected in the bacteriological literature of recent years that it has
come to be believed by many bacteriologists as established truth.

Influence of Conception of Dissociation on Mutation Theory.—But,
along with the general increase in mutation literature following 1907,
there have been interjected into the science of bacteriology other
views regarding the reproductive methods of bacteria which may well
cause us to hesitate in accepting these new forms as actual mutations.
As will be pointed out later in greater detail, these newer concep-
tions of the nature of bacterial reproduction and of the complexity
of forms which it involves, are beginning to lead us away from older
beliefs regarding the simple monomorphic nature of bacteria as a class,
and to supplant them with notions of a plurimorphism underlying some
sort of cyclical development in every bacterial species. With such pos-
sibilities in mind it is clear that the inadequacy of the biotype conception,
as applied by Cole to the problem of bacterial variation, can become
fully apparent only when we are able to recognize the actual thing that
varies. And naturally the same is true of the socalled mutations whose
assumed importance has been reinforced in most recent time through
certain statements coming from both d'Herelle [246,248] and Bordet.[61]

So long as we believed implicitly in the usual textbook statement
that "the mode of reproduction of bacteria is by binary fission," aught
that departed from the regulation cultural type has been classed as an
involution form, a mutant, or a contamination. Usually such departures
from "normal" have been regarded as contaminations and accordingly
discarded. If regarded as "involution forms" or as "Absterbener-
scheinungen" in the sense of Loeffler, they have been tolerated in cul-
tures (and for good reason) but viewed as elements possessing neither
special interest nor significance. If the variation was of sufficient
magnitude and apparently stable, it has been forced into the literature
of the day as a "mutant." Such summary treatment of the unorthodox
members of the bacterial society has undoubtedly done much to keep in
a well domesticated state the cultures which chiefly comprise our pres-
ent laboratory stock-in-trade. As a result of all this, modern bacteri-
ology has become impaled on the monomorphic conception. All of our
laboratory methods have been such as to breed uniformity and constancy.

Dating from the year 1910, however, when Fontès first demonstrated
the filtrability of the tubercle bacillus, or from 1916, when the English

bacteriologist, Hort,[254] first clearly directed attention to the possibility that bacteria possess, upon occasion, other means of reproduction than simple fission, and when Enderlein [159] first introduced his views of bacterial cyclogeny, we have begun to see that some of the apparent departures from the accustomed "types" may possess a significance other than that of contaminations or of insignificant involution forms. Without here entering into the details of what may be involved in this conception (which will be considered later in greater detail), it suffices for the present to say that much evidence exists to show that at least many of the peculiar bacterial forms, now commonly regarded as mutants, stand in some relation to the operation of this reproductive mechanism as it has been revealed, though still vaguely, through the studies of Almquist,[4] Jones,[270] Hort,[254] Löhnis,[310] Mellon,[325] Enderlein [159] and a few others. In other words, the truth is gradually being forced upon us that in bacterial reproduction there is involved a process, perhaps cyclic in nature, and responsible for at least many of the often observed eccentricities in bacterial behavior. This view has been furthered not only by observed changes in the form of bacteria, but also by observations indicating the temporary disappearance of an infecting organism, perhaps for some days, as for example the relapsing fever spirochete in the body of the louse. The organism unquestionably remains in the louse, but apparently in a form in which it is not recognized. Soon, however, it reappears in its "normal" shape. Such observations, coupled with certain observed changes in morphology in the blood of the infected individual, and especially perhaps the finding of the peculiar granular bodies as reported by Wolbach [489] and others, have served to lead several investigators (Leishman, Balfour, Hindle and Fantham) to consider the view that there might exist a "cycle" of development in the life history of the relapsing fever spirochete. The demonstration of the filtrability of this organism by Novy and Knapp [374] in 1906, confirmed later by several others, does much to support this view.

If we may therefore accept for the moment the possibility of this view (namely, that there may exist among bacteria a possibly complex reproductive mechanism, lying quite outside the range of simple fission, and in the course of whose operation new and apparently "unusual" forms of bacteria arise from the parent culture), let us examine the effect of such a view on the theory of bacterial mutation—particularly as related to active microbic dissociation.

If we come to regard the new reproductive mechanism as one of cyclic nature, as depicted so clearly in the Bakterien-Cyclogenie Pro-

logomena of Enderlein,[100] or even if we merely take into consideration
certain different and changing developmental phases in the life of the
bacterium, the problem requires little further consideration; and this for
the reason that we could scarcely regard as a true mutant any organism
transiently typifying a single stage in the life-story of a species. The
important questions to be answered are: does the assumed mutant ever
return to the original form, either directly, or indirectly through an
intermediate type of organism; and does the mutant become the center
of new variations? Although it is perhaps too early to attempt any
final answer to these questions, there can be small doubt that many of
the assumed mutants manifest marked adherence to the newly-acquired
form, and are so reported in the majority of instances. Their stability
is by no means certain, however; and all such reports lose weight in the
face of Jordan's [273, 274] recent results dealing with the reversion of the
R type of B. paratyphosus B to the sensitive form, as well as by the
still more recent work of Soule [450] on the dissociation of B. subtilis.
This question is considered further on another page. On the second
point we know nothing; but it is safe to say that we possess no evidence
to indicate that the new forms have created a new center of variation.

In view of these circumstances it becomes clear that we cannot yet
accept the fact that the many and various cultures reported as mutants
of good standing actually deserve the name. If one wishes to regard
as mutant any culture which exhibits apparent hereditary differences
from the parent stock, persisting in culture for some weeks or perhaps
months—then all of these new discoveries are mutants. It seems to
me, however, that we are justified in applying this term only to those
cultures fulfilling more strict requirements. We should perhaps regard
as mutants only those hereditary variations whose mode falls outside
the limits of the species; and this should be construed as meaning out-
side the range of types represented by the normal changes (cyclic or
otherwise) characteristic of the organism in question. Until, therefore,
we train ourselves to detect the limits and range of cyclogenic variation
characteristic for each bacterial species, we are scarcely in a position to
recognize a mutation when it appears. It is safe to say, however, that
at the present moment we do not know of one unequivocal or authentic
case of mutation among the bacteria.

One may object that, when an organism possessing the features of
B. paratyphosus B becomes transformed into one endowed with the
characteristics of B. typhosus, this must be a mutation. But that will
depend on whether the transformation of the "mutant" can also be

effected in the opposite direction. If that should be found possible, what should be said regarding the integrity of the "species" as now established? Only that they should be consolidated and supplied with different terms of reference. Without meaning to attribute truth to the illustration given above, I do think it probable that, when the limits of normal variation and dissociation of many more organisms are ultimately known, numerous such consolidations will be required. In the meantime we must be skeptical regarding the significance of all "good bacterial species," on whatever grounds they rest; as also regarding the many new types that are appearing, stamped with the "mutation" trademark. Before we can detect the variable we must know from what it varies.

The Bacteriophage as an Agent Producing Mutations.—Regarding the rôle of the bacteriophage in effecting dissociations (and mutations) another point is of interest. In his second book d'Herelle [247] raises the bacteriophage to a point of high biologic significance with respect to its power to determine bacterial mutations; indeed he states, "these mutations are always produced under the influence of the bacteriophage." There might be some misapprehension from this phraseology, but the matter is stated more clearly in his third book in which he remarks (p. 222), "It is indeed probable, as various investigators have suggested, that all of the fixed mutations occurring among bacterial species are produced through the action of the bacteriophage." With the publication of his third book [248] d'Herelle's belief in the mutation-provoking power of the bacteriophage had thus not been diminished in intensity, as is also indicated by the following quotations (p. 225): "In summary, then, the most important facts to be derived from all of these studies is that, exposed to the action of the bacteriophage, bacteria undergo mutations, usually unstable ones, but that these may become fixed under conditions as yet undetermined. These mutations are associated with a state of resistance acquired by the bacteria." And again: "Moreover, a deeper study of these mutations is sure to completely revise our present concept of the fixity of species."

In opposing such views as these, which can serve only to make more difficult the solution of the problem of bacterial species, I can only say once more that the evidence which I have assembled in this work is sufficient to demonstrate that, with respect to its mutation-furthering ability, the bacteriophage can accomplish no more striking modifications than those clearly recognized as occurring in all bacterial cultures when placed under adverse growth conditions such as may be determined by

physical and chemical agents of great variety; and, indeed, no more than bacterial cultures often accomplish for themselves in a small way perhaps, even if grown under conditions which bacteriologists may regard as the most favorable. The important point—indeed the crucial point, which d'Herelle fails to comprehend—is that the cyclogeny of a single bacterial species embraces many strictly normal forms of culture growth, each one of which is endowed with different biochemical, serologic and antigenic characters; and, it may be added, with quite different degrees of resistance to the action of the bacteriophage. To isolate and to study these varied forms is the task of the student of microbic dissociation. In all of these natural cyclogenic changes the bacteriophagic ultravirus, as d'Herelle understands it, plays no part other than its rôle of accelerating such cyclogenic reactions as the culture is already able to accomplish for itself in a rather less energetic manner. That a revision of our conception of the nature and degree of fixity of bacterial species is demanded can scarcely be doubted by any bacteriologist with laboratory experience; but that an analysis of the problem will be furthered through the intercession of the bacteriophage, as such, and of its mythical, mutation-provoking powers, is altogether impossible. The one study that can assist in establishing the new groundwork for this hoped-for revision is the study of microbic dissociation and of the sequence of cyclogenic changes, on which the dissociation must depend; and of which (as we shall without doubt sometime come to see) the phenomenon of the bacteriophage is only a part. In these matters d'Herelle's theory, like modern bacteriology, is impaled upon the old monomorphic conception of the nature of bacteria; and accordingly his views on the nature and origin of bacterial mutations are as valueless as those of all his predecessors who have failed to grasp the true significance of the new biology of the bacteria and its relation to many of the foundation stones upon which d'Herelle's theory of the bacteriophage now rests.

In the exposition of his theory of transmissible autolysis Bordet [61] also finds a mutation-furthering power in the bacteriophage. He refers to the lytic agent as something endowed with powers of "directing the trend of bacterial evolution" and of "controlling the destiny of the species." For him it possesses a sort of "regulating" influence; and he even sees the possibility of its activity, not among bacteria alone, but in the cells, organs and tissues of higher animals as well. But, whether we are justified in attributing to the lytic agent an influence so far-reaching (for the bacteria at least), will depend on whether the "new forms of bacteria" (i. e., the "mutants") generated under its "creative"

stimulus, are actually outside the circle of normal, cyclogenic variation. In earlier pages I have referred to the striking similarity between the R forms of culture arising from active microbic dissociation and the SR (or RR) arising as secondaries to transmissible autolysis; and the analogy is rendered only more perfect when we consider the filtrable forms or the "ultrabacteria" of d'Herelle. We have seen that none of these forms can be regarded as a mutant in the strict meaning of the word. Thus, although Bordet makes some provision for a sort of variability (in degree of sensitiveness) among the cells of his sensitive cultures, his position is not far different from that of d'Herelle when he considers the relation of the lytic agent to mutation. To his conclusions the same answer must therefore be given. While it may yet be shown that the influence of the lytic principle can create forms of bacteria not produced through the mechanism of active microbic dissociation, and lying outside the range of normal, cyclogenic variation in its widest sense, I see no evidence of this at the present time; and, until such evidence is forthcoming, we may well be cautious in attributing too far-reaching an influence to this still unknown agent.

Conclusion.—In concluding this subject of bacterial mutations it may be said that, although new "discoveries" of bacterial mutants are still flowing in abundance into the archives of the science, none of them bear the light of careful scrutiny; and for the reasons that I have already advanced. Firmly adhering to the old monomorphic conception of the nature of bacteria, bacteriologists as a group have not put themselves into a position to recognize a mutation even if it appeared to them. Not until we have become able to recognize the wide range of cyclogenic variation to which all bacterial species are susceptible, shall we be able to detect the permanent departure from the specific cycle. This will take time, and I suspect that it will be many years before we shall be able to speak intelligently regarding bacterial mutations. In the meantime it might be well if the term "mutation," were banished from the vocabulary of the bacteriologist.

16. THE BIOLOGICAL SIGNIFICANCE OF MICROBIC DISSOCIATION

In any natural phenomenon manifested so clearly and so commonly as the dissociative process, and along such parallel lines among various species and genera of bacteria, one is naturally driven to seek for the biologic significance of the reaction—just as the discovery of the common act of conjugation among the ciliated protozoa led to an attempt to develop an explanation. Although many of the physiologic reactions

occurring in bacteria, as well as many biochemical reactions determined by bacteria, seem quite fortuitous, so far as advantage to the organisms themselves is concerned, we should not blind ourselves to the view that some of these reactions are in reality advantageous, while others cannot be regarded as without significance in the struggle for existence merely because we are not yet able to appreciate it. Regarding the varied reactions involved in the phenomenon of microbic dissociation, the first hypothesis which presents itself is, therefore, that it is an adaptive reaction, or one aspect of such a reaction. In partial support of this view there could be marshalled much evidence drawn from the data presented in the foregoing pages to demonstrate that dissociative phenomena of some sort invariably accompany the adaptation of bacteria to new or unfavorable conditions of environment, as for example to the influence of drying, heat, starvation, moisture, antiseptics, oxygen tension, immune serums, the bacteriophage, etc. The newly formed R type, which we believe is a stabilized end product of the dissociative reaction, can live under the new conditions while the old form was unable to do so. Such a view would therefore indicate that a sort of "transmutation," accompanied by the sacrifice of many old characters, is the price paid for survival.

But, on the other hand, we observe that dissociation sometimes occurs in young cultures and often on rich culture mediums affording, as we assume, the best possible conditions for growth. It may occur under environmental conditions which we regard as ideal, while it may appear to be absent on an apparently inferior medium, as I have found to be the case with B. pyocyaneus. Yet we may not be justified in assuming that the sort of food and other conditions which encourage the fastest and most luxuriant growth are always most advantageous for the bacteria, although they may be most satisfactory from the viewpoint of the bacteriologist. Life processes, to proceed normally, must proceed at an optimum rate, and it is conceivable that this rate may be made too rapid for safety as well as it may be too slow. It is possible that the van t'Hoff rule relating to the influence of heat in activating enzyme action may have a parallel in the chemical stimulation of bacterial growth by an "over-rich" medium. In the one instance, as the other, the result may be a depression or annihilation of the active agent. If we take this view, we might conceive that either poor or rich mediums could produce harmful effects to which the same sort of response on the part of the bacteria might be given. This view is supported by

the work of Braun and Schaeffer on B. proteus (starvation) and my own on B. pyocyaneus (rich feeding).

Views Regarding the Nature of Dissociative Variation.—In the literature bearing consciously or unconsciously upon dissociation an explanation has never been attempted; and with few exceptions (Preisz,[393] Hoder,[253] Eastwood[144]) it has not been related either to other phenomena involving the lysis of bacterial cultures or (with the exception of Mellon) to other apparent eccentricities in bacterial behavior. If, however, we regard a marked tendency to variability, and the production of more or less permanent "mutations," as evidences of the dissociative reaction, it has in this manner been linked indirectly with variation-stimulating effects of environment. Gotschlich, for example, in 1903, held that infinite variation is always a characteristic of organisms placed in a new environment, and Kruse (1896) emphasized the fact that unfavorable environment and conditions which permit only slow multiplication (as for instance growth in old cultures) are effective in producing variations. With reference to the relation of dissociation to eccentricities often observed in bacterial behavior, however, the primary studies of Jones, Hort, Eisenberg, Mellon, Löhnis, Löhnis and Smith, Almquist and Enderlein are of marked interest and will be considered further on a later page.

In general, however, so far as the brief explanatory statements regarding dissociation are concerned, they have seldom passed beyond the view that the reactions described are "variation phenomena" or "mutation phenomena" (Neisser,[362] Baerthlein[27, 28]), perhaps stimulated by unfavorable environment. From time to time the older views of Loeffler and other members of his school regarding degeneration phenomena and "Absterbenserscheinungen" have been revived and the variants have accordingly been regarded as merely of teratological significance. Limiting our consideration, therefore, to those more recent studies in which the S and R types have been clearly recognized, we observe various views expressed. De Kruif comes forward definitely to support the mutation view, and bases his opinion on the conception of Dobell[130] who referred to a mutation as "a permanent change—however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations." Amoss also, with reference to his pneumococcus R type, stated that "apparently there has been a genuine mutation." Reimann, also dealing with the pneumococcus R, stated more conservatively that this form is a variant from the S culture type. Griffith was inclined to attribute the dissociative reaction in the pneumo-

coccus to "degenerative changes" in the culture. Krumwiede and his collaborators attributed the generation of R forms of B. paratyphosus and other organisms to "degradation phenomena," and Julianelle [505, 506] has made use of the same manner of interpretation in reference to the R form of Friedländer's bacillus. Regarding the origin and nature of the R form in B. dysenteriae and related organisms Arkwright gave consideration to several possibilities: that they may be "inseparable contaminations;" that S and R forms may preexist in all cultures as elementary forms of the same species; that the S and R forms are merely "modifications" due to the influence of environment; that they may be due to "variation within the limit of the species"; and that they may be actual mutants (in the accepted meaning of the term). Among these possibilities Arkwright came to no definite conclusion but merely stated that "R forms undoubtedly readily arise under artificial surroundings." The circumstance that modifications seemed to arise from so many strains, and so frequently, made him hesitate to evoke the mutation hypothesis. P. B. White in his excellent study of dissociation in Salmonella gave us still another point of view—namely that "roughness" constitutes a deficiency disease of bacteria. He stated: "roughness appears in the light of a disease in which certain antigenic factors may be lost and others altered. . . ." And again: " . . . it is above all probable that roughness is largely conditioned by some nutritive deficiency."

So far as may be ascertained from his major contributions, d'Herelle [248] has not grasped either the fact nor the significance of microbic dissociation, although he has commented on one or two instances which I believe involved this reaction. With reference to the Twort phenomenon and also to the autolytic reaction in B. pyocyaneus he introduces the term "bacterioclysis," a disease of bacteria, which he assumes to be quite different from the phenomenon of the bacteriophage. What sort of a disease is referred to, we are not informed; nor what the cause of the malady may be. But we are left to infer that the condition is at least pathologic and not a normal reaction.

In reviewing these various attempts to appraise the significance of the peculiar reactions in cultures occurring in the progress of microbic reactions in cultures occurring in the progress of microbic dissociation, dissociation, we thus see that they have been related to: (1) "Variation phenomena"; (2) "mutation in bacteria"; (3) degeneration of cultures; (4) "degradation of cells," and (5) "disease in bacteria."

Regarding these views, little need be said. They are all developed out of the false monomorphic conception of the nature of bacteria and of bacterial reproduction. To call these changes "variation phenomena" evades the question and does not further our knowledge in any way. The "mutation view" has been considered in section 15, and as I believe, eliminated as a possible explanation. To view such changes in cultures as manifesting "degeneration" or "degradation," in the usual meaning of these terms, finds little support in the facts observed; and receives, moreover, definite contradiction in the circumstance that the "degraded" forms are commonly more vigorous in growth, and possess greater longevity in the face of unfavorable environmental conditions, than the culture types not so "degraded." To look upon these reactions as due to a "disease of bacteria" ("bacterioclysis"), is perhaps interesting, and it cannot be disputed that any living cell, in whatever phylum, may be susceptible to disease abnormalities in some form; but such conclusions, in the case of the fission fungi, finds no evidence in fact and can only lead us unnecessarily far astray from the point at issue.

In view of this manifest dearth of adequate consideration of the nature and significance of microbic dissociation, and because many aspects of the complex subject of dissociation require some final correlation, I shall attempt, in the present section, to state my own concept of the significance of the reaction; and to introduce, at appropriate points in the discussion, the views of those few authors who have given the matter serious consideration. In so doing I shall attempt to conform, so far as possible, to the experimental data introduced in the earlier pages of this work; and to make it clear where speculation is necessarily called upon to bridge over gaps unspanned as yet by complete or reliable experimental evidence.

A Possible Interpretation of the Dissociative Phenomenon.—In entering the field of inquiry, it must be frankly admitted that we are advancing into an unmapped region but one, the general aspects of which, have been hinted at by a few adventurous workers who have journeyed far from the beaten trails of current bacteriological thought. It is possible nevertheless to formulate an hypothesis which, though having perhaps none too secure a support in facts yet accomplished, may serve to direct exploratory approaches into the deeper problems underlying the acknowledged fact of microbic dissociation. A tentative thesis may therefore be put forward as follows:

Microbic dissociation, as we superficially observe its mechanism and effects, involves the partial or complete elimination by autolytic and

transformatory processes, of the S type of culture when changed conditions of environment become sufficiently prejudicial to a continuance of the same type of growth; and it is accompanied by the generation of new bacterial forms better qualified to perpetuate the stock in the changed environment. In reality and fundamentally, it involves: a process of nuclear reconstruction or rejuvenation effected by the conjugation of certain cells, resulting in the production of zygospores or their physiologic equivalent (and perhaps sexually differentiated cells), and effected at the sacrifice of part or all of the old culture type, which may largely disappear; also a process of division or "fragmentation" or budding of the conjugate cells, resulting in the formation of new elements, cocci or granular bodies, some of which may be ultramicroscopic and filtrable. Some of these filtrable forms are noncultivable; others may continue to propagate in this form (invisible or barely visible growth); while others possess the ability of again entering into the "normal" bacterial aggregate or into some ultimate modification of it (R type). What morphologic type becomes stabilized after its emergence from the dissociative process depends largely upon the extent, intensity or degree of the reaction and upon the selective nature of the environment. It is at this moment that the "evolutionary trend" of the species (in the Bordet sense) might be determined, if it can be said to be influenced at all by such a means.

From this point of view, dissociation as we observe it in its grosser aspects, would be regarded as a normal adaptive reaction made possible through the intervention of a special type of reproductive mechanism in bacteria. This process is doubtless operative at times in all cultures; but, under changed or unfavorable growth conditions, becomes sufficiently intensified to present the more striking manifestations that have attracted special attention, as in the O type cultures. The R forms of bacteria are, therefore, the newly stabilized or partly stabilized types arising from the germination of special cell structures such as the zygospores, or through the production of gonidia. The S types of bacteria sometimes surviving dissociation are, in a similar manner, the remnants of the original culture which have not entered into the modified reproductive process, but have persisted in a limited vegetative reproduction of the same form. To prevent dissociation, therefore, one must eliminate those environmental conditions leading to the conjugative or sexual phases of reproduction and its associated phenomena; and to stimulate dissociation one must bring about those conditions favoring conjugative and related cell activity and its products, living or non-

living. In transmissible autolysis (passive dissociation) whatever thing first precipitates the reaction, the substance that perpetuates it in cultures is manifestly a product or a form of the bacteria themselves; and probably a result of conjugative or other related cell activity. It may be a metabolic product, but it is more likely to be a living, ultramicroscopic form of the organism itself, endowed with greater resistance and able to produce in the parent culture centers of proliferative growth. This is at once followed by lysis, which is accompanied by liberation of a fresh generation of filtrable forms.

It is to an hypothesis of this sort that I am led from a consideration of such facts as I have presented earlier in this work. Since such a conception brings into relation several phenomena which have usually been regarded as distinct and unrelated, the proof of an hypothesis of this sort is an undertaking of considerable proportions. But there already exists some evidence that supports at least certain phases of such a view; and there also exist certain theoretical considerations and deductions which, though scarcely serving as evidence, are not entirely without force.

The Problem of Growth Cycles.—Regarding the observed facts which support such a view of the existence among bacteria of some form of reproduction involving a reproductive process quite different from binary fission and underlying the phenomenon of dissociation, one naturally goes back to the primary observations of Hort[254] and Enderlein.[160] It is now more than ten years since the former investigator in England and the latter in Berlin made their first fundamental observations in this field and reported on the complicated "life-story" (to use Hort's words) of B. typhosus (Hort) and several other organisms (Enderlein). It was at this time that Enderlein introduced into bacteriological literature the term, "Cyclogenie" (cyclogeny), referring to the series of progressive and degressive changes through which an organism passed in its life-cycle, departing from, and returning to, its basic morphologic form. The facts developed by Hort in his first and later studies, sufficiently remarkable at the time, were amply confirmed by Leishman, Adami and others appointed as a special committee of the British Medical Board to review his entire work. Hort's essential conclusion was that there existed in B. typhosus a form of reproduction, hitherto unrecognized, presumably involving stages of conjugation and endosporulation. He traced the cell changes in warm-field studies and presented a large volume of clearcut photomicrographic evidence. The views of Hort were manifestly a continuation of the conception of the

"Entwicklungscyclus" introduced into bacteriology first by Fuhr-
mann [186] in the year 1907 and developed to a much greater extent by
Enderlein in the years from 1916 to 1925. Evidence of a sort similar
to that of Hort was supplied by Löhnis in his monumental volume on
variation in many bacterial species; and in later years by Mellon in his
long series of papers, by Almquist,[4, 5] Lieske [303] and others. Ender-
lein [160] in his comprehensive work (Bakterien-Cyclogenie Prologo-
mena *) of 1925, not only seemed to confirm much of the work of
these earlier workers, but also introduced for the first time certain
apparently significant data bearing on a distinct mode of sexual repro-
duction by bacteria, lying outside the simple amphimixis obtained
through resort to the production of zygospores and endosporulation. I
believe that the studies of these major workers have not only been of
value in themselves but have served the purpose of lending greater
significance to the host of minor observations over a period of many
years dealing with phenomena that are manifestly closely related; and
especially with reference to conjugation and to gonidia formation, as
observed by Cohn in the early days of bacteriology. To these observa-
tions should be added the phenomenon of reproduction through the
agency of symplastic structures, first called attention to by Jones [270] in
1913 and mentioned again in 1920. This observation has apparently
been confirmed by both Löhnis and Enderlein.

Such reports as those mentioned above and the radical inferences
that have led from them have, for the most part and until quite recently,
however, fallen upon the ears of an unsympathetic bacteriological world.
They are sufficient, however, even if theoretic considerations were not
also in its favor, to establish not only the probability, but as I believe
the fact, that conjugation, or some physiologic equivalent of amphimixis,
accompanied by a form of endosporulation, gonidia formation and per-
haps budding, actually occurs more or less regularly in the life of the

* Enderlein's comprehensive work, correlating and interpreting many earlier observations,
as well as adding many that are new, and dealing with the assumed cyclic changes in bacterial
reproduction, is unquestionably the best presentation of those cell changes (comparative bacterial
morphology), some of which without doubt underlie the dissociative reaction. Enderlein, how-
ever, concerns himself with the cytological aspects rather than with the colonial and cultural,
biochemical and serological, features of bacterial variation; and in this respect has attacked
the problem of bacterial life cycles from the under side, so-to-speak, much as Hort, Löhnis
and Mellon have also done. It is impossible within the scope of this review (which is con-
cerned with the broader and admittedly more superficial aspects of the dissociation phenome-
non) to give an adequate account of the complex comparative cytology of bacteria as presented
by Enderlein. Suffice it to say, that the subject under his hands becomes involved with the
description of cell phenomena and developmental stages which few bacteriologists have ever
seen, much less followed; and for which he coins a large vocabulary of new and unusual
terms. As such studies are now progressing among bacteriologists at large, it will probably be
many years before a true appraisal of Enderlein's contribution can be made. In the meantime,
however, we may regard with no little admiration his manifestly careful and sincere attempt to
put some degree of order into the at present chaotic state of bacterial cytology. I believe that
Enderlein has blazed a trail which, at least in the main lines of advance, other bacteriologists
sooner or later are sure to follow.

majority of bacterial species. As for true sexual reproduction, involving the highly specialized sex cells (spermites and oites of Enderlein) we may perhaps be justified in holding our conclusions in reserve, although I do not regard the circumstance as improbable. The pictures presented by Hort, Eisenberg, Löhnis, Mellon, Almquist, Enderlein and others are in almost perfect agreement and can scarcely have any other interpretation than that first proposed by Fuhrmann twenty years ago, and by Hort almost a decade later. Although I have not followed the successive cell changes on the agar block as was done by Hort, I have seen in fresh and in stained preparations of B. diphtheriae many of the same elements. I have also observed repeatedly that the clearest pictures of zygospore-like bodies, containing distinct chromidia, occur in preparations taken from actively dissociating cultures; and that the best pictures of the stabilized secondaries, on the other hand, are derived from cultures in which the dissociative process has passed the height of its reaction. Moreover, I have found it distinctly observable that the prelude to dissociation in several species (B. coli, B. typhosus, Bact. pneumoniae, B. diphtheriae) yields much the same microscopic picture as that observed at the beginning of the new reproductive stages outlined above. It is namely a proliferative growth accompanied by a tendency to develop granular or coccus forms (gonidia) within the rods; also to form long, and often beaded filaments, frequently branched as detailed by Gardiner[195] in his studies on "three-point multiplication." This sign was first mentioned by Hort as preluding what he termed, for B. typhosus, the "reproductive explosion;" and was again emphasized by Mellon as a phenomenon clearly announcing the initiation of a new reproductive phase in the culture, marked chiefly by the production of conjugative cells and subsequently of zygospore-like bodies; and often terminating in a new or modified culture type.

Not only in B. diphtheriae (Park 8 strain) but also in B. malleus and in Streptococcus viridans, I have been able to recognize the large pale spheres ("giant cocci") measuring up to 6 or 7μ in diameter and containing chromidial bodies to the extent of two to twelve as often described for the diphtheria bacillus, as described for the diphtheroids by Mellon,[326, 327] for B. coli by Mellon,[330] for B. typhosus by Eisenberg[147, 154] and others; as described and pictured for the same organism by Hort,[258] and by Löhnis[310] for many species, by Jones[270] for azotobacter, as most recently recorded by Enderlein[160] for Vib. cholerae and as followed in their subsequent development particularly by Hort, Mellon and Enderlein. In Eisenberg's case these forms were produced

during the apparent dissociation of the typhoid bacillus under the
influence of normal, but germicidal human serum.   In some of Mel-
lon's cases ascitic fluid in the medium seemed to have an influence;
while most of Hort's special forms arose on an alkaline agar.*   I
believe there is no other logical explanation for these frequently
described "involution" forms than that they are related to a form of
reproduction involving the production of gonidia, also conjugation and
zygospore formation.   Regarding the existence of a distinctly sexual
form of reproduction ("oites" and "spermites") as pictured by Ender-
lein,[160] there is less certainty.   Whether, as suggested by Mellon and by
Hort, the zygospores are the mother cells of the filtrable forms of
bacteria, for the existence of which in many species sufficient evidence
may now be said to exist, or whether the filtrable bodies are the micro-
gonidia or the "gonites" as suggested by Enderlein, Almquist[3] (for
B. typhosus) and Mellon[337] (for B. fusiformis) cannot at present be
stated.   Nicolle,[368] however, has voiced the possibility of the origin of
the filtrable viruses from bacterial cells, a conception which appears to
be gaining ground through the circumstance that filtrable forms are
rapidly being discovered for many bacterial species.   But, whatever the
actual significance of these minute bacterial forms may eventually prove
to be, we may be certain of three things: they occur regularly, con-
sistently and in great numbers in many bacterial cultures under certain
conditions of growth and at a certain stage of development; they do not
always long endure as such, but after a brief development often dis-
appear, apparently passing into other developmental stages; and
although they may closely resemble certain artificial structures on the
slide, they are not artefacts.

Under the present circumstances, when one is somewhat at a loss
to shape a difficult hypothesis, there may be some justification for add-
ing to what has preceded certain theoretical considerations which favor
the concept of the existence of at least a conjugative reproductive pro-
cess among bacteria.   These considerations relate to two fundamental
aspects of protoplasmic behavior: the necessity of some form of reju-
venation for any continuous stream of protolasm; and the possibility of
regeneration from nuclear fragments or chromidia.

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* It is scarcely feasible in the present work to undertake a review of the many interesting
details of culture and cell modification reported in recent studies.   Many of the facts still
require confirmation, but those interested in pursuing the subject further are referred to the
series of papers by Mellon, in the course of publication, and to the comprehensive work of
Enderlein in his Bakterien-Cyclogenic Prologomena (1925).   See previous footnote.

In many respects it is a most remarkable circumstance that bacteriologists as a rule have been so long content to regard with equanimity the assumed absence of any rejuvenating mechanism in the germplasm of bacteria, while it is observed, at least as an occasional necessity, in nearly all other forms of germplasm with which biologists are familiar; and may even be regarded as a universal need of living things. And the same holds true for some form of nuclear "fragmentation" and subsequent cytoplasmic regeneration observable in nearly all forms of plant and animal life. It is true that in some yeasts an exclusively asexual process of reproduction seems to obtain permanently, but this fact is not conclusive. Respecting this point, we may perchance do well to remind ourselves of the history of the conception of sexual reproduction of the yeasts as it concerned the botanists. At that early date when sexual phenomena were first described for yeasts they were regarded as very rare occurrences—just as most bacteriologists believe at the present time with reference to the bacteria. But it eventually became recognized by the botanists that such sexual phenomena were in reality very widespread. Each succeeding year has added fresh instances and new, important data on the reproductive mechanism until, at the present time, some botanists and mycologists entertain the view that sexual reproduction, though of a very primitive type, may occur, at least at some time and under favorable conditions, among all yeast species. Such a history of a biological conception with reference to organisms showing many characteristics in common with the bacteria is not without significance; but still the truth is that, among bacteriologists, nearly all have united in denying to the bacteria as a class, presumably because of their minute size and structure, the possibility of participation in a form of reproduction and nuclear rejuvenation which has long ago been clearly proved and accepted in other simple living things; and particularly in forms closely related to the bacteria.

But, merely because bacteria are minute and apparently simple in organization, is no logical reason for attributing to them a reproductive mechanism that is correspondingly simple; and for denying to them the possibility of sharing with related forms a reproductive process that is intricate and difficult of clear observation—detectable only by those who have the patience and perseverance to search the most diligently. In a way, it seems to me that there may be less justification for our present belief in the existence of the filtrable virus as a distinct biological entity than for our acceptance of a view favoring the existence in bacteria of a reproductive mechanism making possible nuclear rejuvenation;

and of a process in the final explanation of which, the nature of the filtrable virus itself may ultimately find its solution. Bacteriologists as a group, ardent in their support of the living, self-propagating nature of the ultravirus against current biological views which bound all life by the organized cell, have themselves, until most recently, failed to greet with appreciable understanding the only genuine attempts through which it seems possible that a fuller knowledge of the ultravirus might be derived. I am convinced that many fundamental problems in bacteriology today, and among them those of microbic dissociation, transmissible autolysis and the origin of the filtrable virus, will receive their solution only when we shall have taken up the task of studying the actual mechanism of bacterial reproduction from a broader biological viewpoint than that which has characterized the majority of work on bacterial reproduction and variation up to the present time.

Relation of Microbic Dissociation to Reproductive Phenomena (Bacterial Cyclogeny).—If, therefore, we may tentatively accept the view that some sort of a cyclical reproductive process, involving conjugative reactions, exists for bacteria (perhaps in the sense indicated by Enderlein's [160] term "Cyclogenie"), the question must arise: What is the actual relation of microbic dissociation and of the S, O and R forms of culture to this reproductive system? It can only be answered at present that we do not know. In the light of many observations, however, a suggestion may be hazarded although it may be long before either its truth or falsity can be proved. This view may be stated as follows:

When bacteria grow on a medium to which they are well adapted they multiply, at least for the most part, by a simple vegetative process, binary fission; and in consequence present collectively a high degree of uniformity in appearance, as well as in most of their biochemical, antigenic and serologic reactions. This is the normal S type, carrying the double antigen, S (in preponderance) and O (in smaller measure).

When, however, the environment surrounding these organisms changes, and particularly when it changes in a manner unfavorable to the continued vegetative growth of the same culture form, the first effect of such a change is to set into operation (or at least to intensify) in the culture a new reproductive process including opportunity for nuclear reorganization, and looking forward to an adaptation to the new conditions of growth.

This process of nuclear reorganization involves primarily the suppression of the vegetative reproduction by the usual fission and the bringing to the front a form of conjugative reproduction calculated to afford a fresh basis for variability and consequent adaptation. This culture stage is characterized by the generation of filamentous forms, beaded (gonidial) and "involution" structures, and zygospores or analogous bodies produced by conjugation. This varied form of culture represents the transitional or O type. It carries as its fundamental antigen the O, sometimes pure, but often mixed either with S or R.

The O antigen, at least in certain of the transitional forms, is characterized by heat stability. The same is true of the R antigen.

From the zygospores or related bodies embraced in the O culture there arises, as a result of budding or endosporulation or in some still unknown manner, a new generation of cells or other bodies, some of which may be filtrable, but many of which are endowed with a higher degree of adaptability to new growth conditions than was the parent form.

Although these derived bodies (cells or invisible elements) may, under favorable conditions, hold to the same type of growth through several or perhaps many generations, they gradually conform to the demands of the new environment and become temporarily stabilized in a new morphologic and biochemical type the nature of which is determined, within limits, by the existant environmental conditions, as well as by the specific cyclogeny.

The gradual stabilization of the new form is usually, but not always, accompanied by a return to a vegetative mode of reproduction and by a corresponding suppression of sexual or conjugative reproductive methods which were instrumental in its generation. The new culture type, if perpetuated in the new environment, again becomes uniform in composition and, within limits, constant. It now possesses the new heat stable antigen, R; and may have generated a little of the antigen, S. It is not, however, a true mutant since it carries the potentiality for "reversion."

In considering the modified nature of the O and R types of bacteria and their apparently frequent stabilization in culture, the question will undoubtedly be asked, whether these changes are all actually related to the genetic mechanism or whether some of them may be "impressed" variations; or even forms possessing only teratological significance. It is impossible to answer this question with any degree of finality at this time. I believe however that the majority of evidence favors the former view, although actual cases of polymorphism may exist—(Mellon [341]). Of such evidence I believe the most significant is the following. While many of the O and R culture forms are known to arise under conditions which appear to enforce dissociation, such as heating, contact with antiseptics, drying, submission to immune serum, etc., it is an important fact that no variant cultural, biochemical or serologic types appear to be produced in this manner that cannot also be discovered in cultures existing under conditions of growth which we commonly regard as "normal" and physiologically the most favorable; and under conditions far removed from any "enforcing" influences, so far as we can observe. In other words, we can note no significant qualitative difference between the effects of "spontaneous" dissociation and "enforced" dissociation so far as the diversity and nature of the variants are concerned. This circumstance would seem to relieve such variants from the suspicion of being "diseased," or of having been produced as "pathologic" forms under the pressure of unfavorable environment, although we know

well enough that such an environment produces these results most
quickly and most markedly. It must be freely admitted, however, that
there still remains a question as to the extent to which environment may
modify any single stage in the cyclogenic series. Although the majority
of the variants that have been described in the literature are undoubtedly
produced through the agency of the genetic mechanism, "impressed"
variations are as yet by no means ruled out of the field of bacterial
variation, and future workers will have the task of attempting to dis-
tinguish between these possible forms of culture and the true cyclogenic
types.

What is the "Normal" Bacterial Type?—From the foregoing con-
siderations one may be inclined to ask—what is the normal bacterial
type? Or even—is there such a thing as a normal bacterial type? It
is true that through long years of laboratory study we have come to
regard as the normal type that form of the organism which grows best
for us on artificial culture mediums. But, even in this regard, there
have always been grounds for confusion, since it has clearly been recog-
nized that the kind of culture obtained on one medium may be different
from the same culture grown on another medium. Even the "same"
medium, in dry or moist states, may produce deepseated changes in the
culture. What, then, under these varying conditions is the "normal?"

Without the need of multiplying the fallacies of many of our old-
time arguments, I believe that a careful consideration of the data
assembled in the preceding pages tends to establish the view that "nor-
mal culture" or "normal type," in the absolute meaning of these terms
and as commonly employed, is something of a myth. The stock typhoid
organisms which we maintain in the laboratory are not the same in all
respects as those found in the blood or intestines of typhoid patients at
the time of isolation; nor necessarily the same as those isolated from
the urine or gall bladder of typhoid convalescents. What, then, is the
"normal" typhoid organism and culture? The same question may
legitimately be asked in the case of other pathogenic bacterial species;
and also, though perhaps to a smaller extent, in the case of saprogenic
forms. Shall we regard as "normal," the disease form, the convalescent
form or the old laboratory form? Also, what shall we say regarding the
"intermediates" and the filtrable forms? We have become accustomed,
I believe, to regard as normal that form of the organism or culture
which occurs most prominently in the sort of reaction or environment
that interests us most; and bacteriologists for the most part have been
chiefly interested in their culture tube collections. It is these organisms

—often tame, domesticated things—that have been set up as "types" and as standards of normality. It would seem to me much more accurate, however, to refrain from speaking of normality in such an absolute sense, but to regard a culture as normal relative to a given condition of environment; or from the viewpoint of a definitely conditioned reaction. Thus in typhoid we might well refer to the "normal laboratory type," to the "normal disease type" or to the normal type of growth on phenol agar, or at 42 C. or in homologous immune serum—meaning in all these instances the form of typhoid culture which experience has shown usually to be correlated with them. In this manner many years ago certain German writers ceased to speak so much of normal and abnormal cultures of B. anthracis as of the normal, "tierische" or the normal, laboratory forms.

All this must not be construed as meaning that we may not have normal or abnormal species or races of bacteria. It means only that, when we speak of normality or of abnormality, we must make our comparison, not between different stages in the cyclogeny of the strains, but between corresponding stages. In other words, we must compare S type culture with S type, R type culture with R type, and intermediate with intermediate—insofar as we are able to recognize these forms. And to succeed in recognizing them, must be our first business if we are going to attempt comparisons. I have no doubt that some of the greatest sources of confusion and error, not only in systematic but also in applied bacteriology (particularly in serology and immunology), are to be found in our well entrenched habit of attempting to make comparisons between fundamentally different things—that is to say, between cultures in different cyclogenic phases of development. I believe, moreover, that until this viewpoint is changed no successful advance can be made through the commonly used methods of classification of bacterial species or races or strains by seeking to establish "serologic types" as an end in themselves. For advancing the fundamental problems involved, such limited methods are, at the present stage of our knowledge of cyclogenic variation, of little value, except perhaps (if need be) to emphasize more quickly their own futility. Indeed, it seems to me that we have arrived at a point in the development of the science of bacteriology where much work on elaborate superstructures must be suspended until we have made more stable the foundations.

Conclusion.—In concluding and summarizing this section we may return to our original question—What is the biological significance of dissociation? In the present state of our knowledge, although there exist sufficient data from experiment and observation to formulate an

hypothesis which no doubt contains some elements of truth, in its finer details it still must be left an open question. At the same time it is clear that the wide extent of its occurrence in diverse bacterial groups, the orderly and usually parallel nature of its manifestations, and the fairly constant nature of its effects upon the cultures concerned, can lead us only to the conclusion that it is not a meaningless reaction, but one possessing considerable significance in the life of the culture if not in the species. If this is true, the significance, whatever else it entails, must be one of a reproductive nature. When, moreover, we examine critically the morphologic changes in the cells occurring during and just preceding the dissociative reaction, we are forced to the view that the reproductive significance of this phenomenon must concern a generative mechanism quite different from simple binary fission in the traditional sense; and something far more complicated. And when, finally, we observe that the microscopic pictures preceding or accompanying microbic dissociation reveal an unusual wealth of those peculiar cell structures which have been recorded persistently for more than two decades in the handful of studies either suggesting or definitely dealing with new reproductive mechanisms among bacteria, we have added evidence that microbic dissociation, as an adaptive reaction, stands in close relation to a type of reproduction about which we as yet know little. More than this cannot be said at present regarding its biological significance. But, if we can accept this much as a working hypothesis, it can scarcely be doubted that the important facts, in their proper relations, will follow in the course of further persistent inquiry.

17. GENERAL CONCLUSIONS

From the varied assortment of data on experiment and observation which I have brought together in the foregoing pages of this review, I believe it becomes clear that many important problems in modern bacteriology and pathology have their roots in the phenomenon of bacterial instability, and especially in that aspect which has been termed microbic dissociation. We begin to appreciate, moreover, that this phenomenon, far from being a sign of chaos, as many have believed, is in reality merely the necessary manifestation of certain more or less orderly processes that are correlated with a physiologic and reproductive mechanism the nature and significance of which we are just beginning to observe and, perhaps to a limited extent, to understand. In the operation of this mechanism we can scarcely doubt that there are concerned significant issues dealing with bacterial relationships, with serological

behavior, biochemical reactions, immunologic reactions, with pathogenicity and virulence; and, in all probability, with the problem of transmissible bacterial autolysis as well.

Regarding the relation of dissociation to the systematic aspects of the science of bacteriology it may be safely predicted that the time will soon arrive when, in discussing the nature of bacterial cultures isolated from infected tissues or maintained in laboratory stocks, it will not be sufficient merely to name the organism. The specific name of a microbe, as names now stand, may mean little; for it is becoming increasingly clear that we shall never know what a bacterial species really is until we acquaint ourselves with the outermost limits of its variability; and this must mean not merely its "normal" vegetative form, but all of its dissociated types or cyclogenic stages as well. Our exact knowledge of an organism may be slight even after we have noted its morphology, recorded its measurements, registered its reactions, ascertained its common serologic and antigenic characters and given it a name. And this is because these superficial considerations, based only upon the "normal form," give us slight understanding of its potentiality for, or mode of, variation under changed conditions of growth. We do not perhaps see the bacillus masquerading as a streptococcus, the branched filament hidden beneath the cloak of the common rod, the true meningococcus in its "giant coccoid" state, or the granule of the invisible virus latent amongst the microscopically visible elements. The truth we shall eventually come to, however, is that the free-living microorganism is potentially a kaleidoscopic thing, in which the power of responding successfully to a changing environment by alterations in body state, both morphologic and biochemical—and even by self-destruction, if need be, in order to generate another and more stable type—stands as its one most important attribute.

It is of course quite true that we can remove an organism from its customary environment, confine it for years in tubes, and enforce upon it thereby a certain kind of stability. Some such "domesticated" cultures, particularly saprophytes, may under such conditions retain perhaps indefinitely their original proclivities, while others, notably pathogenic species, may quickly lose much of their ancient heritage. In so doing, however, they become increasingly amenable to consistant laboratory findings; and have therefore become the favorite subject-matter of the systematists. Long ago, Darwin recognized this situation with respect to variation in higher forms, and consistently avoided the

impasse to his study of variation determined by too continuous an exist-
ence of experimental stock in an unchanging environment.

But, outside the field of cultures possessing such enforced stability,
it must be admitted that most cultures, when first secured from their
natural habitat and placed upon the usual culture mediums, possess
great potential variability. Each apparent species is surrounded by its
small group of satellites to each of which we unwisely attempt to assign
a classificatory niche. We have meningococcus and pneumococcus types
which we usually number in Roman; paratyphoid and streptococcus
types for which we employ the Greek; B. coli and Bact. aerogenes types
for which we employ the small Arabic; B. dysenteriae types to which
we assign the name of the discoverer; and still other "types" which we
have difficulty in figuring out at all. But such procedures do not yield
an advance toward a clearer understanding of the fundamental nature
of the species concerned, nor of their actual genetic relationships.
Although sometimes of practical value, they are for the most part make-
shifts only; and we should not long be content to permit their endur-
ance without demanding a more complete recognition of their meaning
and significance.

What we ought to wish to know about these cultures is, for instance,
how the various types of pneumococci, of streptococci, meningococci,
of B. coli, of the paratyphoids, of dysentery and other bacteria, have been
(we might even say "are being") formed. Are types 2 and 3 of the
pneumococcus variants of type 1; and, if so, under what conditions do
they arise? What genetic relationship or sequence in origin exists
between B. coli a, b and c? And the same for the paratyphoids, the
diphtheria bacilli and other bacteria. These are questions of practical
significance to be answered; but it is difficult to see how they can be
answered so long as we concentrate all our attention on methods of
classification rather than on the one thing that is most essential—the
problem of the nature and origin of variations. This single problem,
for higher animals and plants, is the stumbling block in the path of
evolution inquiry; but we are not so certain that the difficulty exists, in
the same way at least, for bacteria. Although we may not learn how
to create a "species," there is no class of organisms more favorable than
bacteria for studying the possible influence of environment in determin-
ing the trend of hereditary variation.

One object of this discussion, however, is not only to emphasize our
present inadequate conception of the nature of bacterial species and of
the systematic relationships of the bacteria, but also to point out cer-

tain observed grounds for microbic instability and to suggest possible methods of reorganizing the system. It seems to me that this must consist first of abandoning, for the present at least, our vain attempts to perfect schemes of classification. This seems logical, especially in view of the fact that we do not yet know exactly what it is that we have to classify. Secondly and more important, it consists in initiating a somewhat new branch of bacteriological study in which we shall strive to recognize bacterial species relationships, not by a comparison of isolated single cultures of this or that, but by a study of all the various types or stages comprising the cyclogeny of the species in question—a difficult but necessary task. I believe it is only in this way that we shall come to understand the limits and the relative significance of cyclogenic, fortuitous and impressed variations; and so be able to recognize the bacterial species in its entirety. Bacillus diphtheriae, as assumedly typifying a definite bacterial species, should eventually come to mean to us —not merely a rod of fixed size and shape (possessing certain constant biochemical, serologic, immunologic, and staining reactions, by means of which it can conveniently be recognized and cataloged), but in reality a host of things, which we must be able to recognize individually and collectively before we can affirm that we know the "species." Bacillus anthracis, as a "bamboo rod" of certain shape, size, colony form and pathogenicity, has occupied the attention of countless bacteriologists for fifty years; but who at present would dare affirm that we know the anthrax species? Our ignorance of some of its most intrinsic characteristics is nearly as great today as it was half a century ago. In these species, and in others, important problems are awaiting solution.

Regarding the mode of approach to the species problem, as also to several others, I believe the simplest and most direct way in which the average bacteriologist can render service is to attack the subject from the viewpoint of microbic dissociation, as the outstanding features of this phenomenon have been outlined in these pages. And this refers mainly to an attempt to recognize and study cultures arising from the two chief colony types, S and R, together with such intermediates (O) as may be observed. While such a mode of approach does not necessarily bring us face to face with the biological realities underlying dissociation itself, it should serve to make available a mass of evidence which may not only prove of value in useful bacteriological procedures, but also have the merit of indicating more clearly what the exact nature of the more remote problems underlying dissociation really is. In facing such a complex question it may be best that the beginning work should

not dig too deep.   Here again, perhaps, we must ascertain more clearly
just what we have to explain before we attempt to explain it.   On the
other hand, much may doubtless be gained by those few adventurous
workers, such as Mellon and Enderlein, who are engaged so-to-speak
in attempting to solve the problem from the bottom up.   Theirs is at
present the more difficult task.   But, when the two groups ultimately
meet, it may be predicted that we shall have an understanding, not only
of the significance of microbic dissociation, but also of still largely
unknown reproductive phenomena among the bacteria.

For a partial indication as to what success may be expected to arise
from such endeavors one needs only to refer to such studies as those of
Stearn, Gratia and Mellon on B. coli; of Mellon on the diphtheroids;
of de Kruif on the Pasteurella; of Weil, Felix, and Braun and Schaeffer
and others on B. proteus; of Bernhardt on B. diphtheriae; of Cowan on
the streptococci; of Stryker, Griffith, Reimann and of Amoss on the
pneumococcus; of Goyle on B. typhosus and B. enteritidis; of Balteanu
on the cholera vibrio; of Enderlein on the cholera vibrio and the diph-
theria bacillus and of Julianelle on Friedländer's bacillus.   All of these
studies begin to show, for the first time and in one way or another,
how, in what sequence, and under what conditions of environment,
cyclogenic variations have been, and can be, produced.   Whether
such changes in general, or any stage in particular can, by artificial
means, be made permanently hereditary, may be left an open ques-
tion.   Undoubtedly many of them possess remarkable stability.   We
see that the geneticist, working with multicellular forms, has been
able, by the manipulation of his unit characters and with the aid of the
Mendelian principles of segregation, as also of sex-linkage, to blend
these available units into new configurations; but he has not been able
to synthesize a new species.   It is permissible to believe, however, that
bacteriologists, working with a less differentiated and more impression-
able protoplasm, may yet be able to produce from known stocks, new
bacterial forms possessing at least the equivalence of what have hereto-
fore been termed species; but for which new designations would be
required.   At the present time our convictions are such that the con-
ception of bacterial hybrids as introduced by Almquist[6] in 1924 appears
to most bacteriologists as the height of absurdity.   While it is quite true
that his remarkable results, like those of Castellani,[91] may be explained on
other grounds than that of an actual crossing of species, and that all
possible attempts should be made so to explain them, it also may be
remarked that their mirth-provoking power is today much less than it

would have been a decade or more ago. It must be frankly admitted that we still have little intimate knowledge of the private life of the bacterium.

In this connection there should be mentioned another field in which I believe microbic dissociation may eventually play an important part. This is in connection with the rickettsial bodies, both pathogenic and nonpathogenic. In the study of these elements, whether intracellular or extracellular, there is now ample support for the growing suspicion that they are related in some way to the better known bacterial forms. This is perhaps indicated most clearly in the case of Rickettsia prowazeki and B. proteus X19 of Weil and Felix. The results of many histologic, serologic and immunologic studies seem at present to combine in suggesting that the typhus fever rickettsiae may be a dissociated stage of the proteus organism. With further inquiry, as the earlier histologic approaches give way to cultural studies, it seems probable that new and significant facts bearing on the dissociative reaction will be brought to light.

That the brief knowledge already available regarding the dissociative reactions among disease-producing bacteria is even now in a position to give us a more exact and critical view of certain serologic and immunologic reactions there can be no doubt. It not only makes clear many previously observed inconsistencies, but helps both to indicate and explain the limits of serviceability of many serologic reactions as used for diagnosis. In addition it presents the most tangible basis for an understanding of the confusing data that have gathered about the subject of the "double" and "single" antigens, as well as the "major" and "minor" agglutinins. In relation to still other methods of bacteriologic diagnosis (cultural, microscopic, biochemical) it affords a new and important point of view by virtue of which we are in a position more carefully to observe and more intelligently to interpret the results of bacteriologic examinations. Although its bearing upon immunological theory and practice is as yet hardly touched upon, it already shows us one possible reason for frequent failure to obtain expected results from current immunological procedures; and thereby opens the door to a perhaps helpful reconsideration of several significant problems relating to vaccine therapy in man. Regarding the rôle of pathogenic organisms in infections, a knowledge of dissociation phenomena offers, for the first time, a rational and exact basis for developing virulence by individual colony, rather than by mass culture, selection. It affords us, moreover, a closer view of the origin and the far-reaching significance

for serology and immunology of the socalled specific soluble substances of bacteria. Through the now more clearly recognized mode of action of immune serum on bacterial cultures in vitro it offers a new conception of one perhaps highly significant mechanism of humoral and cellular defense in the living body against invading micro-organisms; and thus may afford an explanation, heretofore lacking, regarding the actual mode of protective action of the bacteriotropic antibodies. In view of the dissociation-furthering influence of high growth temperatures on several pathogenic bacterial species, microbic dissociation may supply the grounds for a better understanding of the rôle of hyperthermia in infectious disease; also, regarding the the cause of relapse in fevers as being related to incomplete dissociations, or to the reversion-provoking power of R type immune serum, as demonstrated by Soule for the nonpathogen, B. subtilis. It fixes in our mind, moreover, the vast, but heretofore unrecognized, importance in the treatment of disease, of the control of the cyclostage in the individual case, as already emphasized by Enderlein; and persuades us that it is toward this end, rather than toward the often impossible direct elimination of the infecting organism, that we should look.

In epidemiology our knowledge of the dissociation phenomena in relation to specific, pathogenic, bacterial species is certain to cause a revision of many of our older views, especially those relating to the "carrier state." It may be that we shall sometime learn that the progress of the epidemic is merely the gradual development of the infective agent into its virostage; and that the remission of the epidemic is due to the passage of the infecting agent through the virostage, and beyond it, into a nonvirulent form. Indeed, we may conclude by saying that there are few fields in either systematic or applied bacteriology that are not in a position to be illumined and augmented through the knowledge that has been and will be gained regarding microbic dissociation.

Regarding the joint relation of microbic dissociation and the phenomenon of the bacteriophage to microbic instability, we have seen that there are good reasons for suspecting that both of these reactions are inseparably united in the reproductive mechanism of the bacterial cell. It seems to be the "normal" functioning of this mechanism, operating in a favorable, and one might add accustomed, environment, that determines traditional microbic stability; while it is the "abnormal" or "pathological" (Bordet) functioning of the same mechanism that conditions mutation-like changes, accompanied by lysis of varying grades and possessing great significance in the life of the culture—perhaps in

the life of the "species." At the same time, we may not be quite justified in regarding these dissociative transformations, (whether active or passive) as "abnormal" or "pathological" in any other sense than that they may be manifested to an unusually high degree or that they may be forced—sometimes intentionally (as in provoking transmissible autolysis), and sometimes unintentionally (as in "spontaneous" dissociation). We have seen that there is much evidence that the reactions of microorganisms are much the same in both; and that these reactions possibly represent the only means by which bacteria are able to insure the survival of their germplasm in the face of age or unfavorable environment. In this presumably adaptive reaction we may also eventually come to see that supremely important, but still largely unrecognized, events in the life of the bacterial species are occurring during that stage of the dissociative reaction lying between the disappearance of the old form and the generation of the new.

When, moreover, we pass in review the many and varied manifestations of autolysis and regeneration, whether in appearance "transmissible" or merely "dissociative," and observe the frequent connecting links between these reactions, we can scarcely doubt that we are dealing in all instances with a single biologic phenomenon which, depending on the speed of reaction and correlated features, gives us the varied lytic manifestations that we are trying to explain. May it not be probable that some organisms, well adapted to their environment, live and die without presenting, or presenting only obscurely, outstanding lytic and regenerative tendencies? Or that others of different constitution give us the moderate changes seen in the slow-going, active dissociations? Or that others, unstabilized by new environmental conditions, react by giving the rapid dissociations seen in the typical "suicide cultures?" Or that still others afford a sort of dissociation that is transmissible through the agency of filtrates (rather perhaps than from cell to cell) and characterized by great suddenness, as typified by the classic bacteriophage phenomenon of the Shiga dysentery bacillus?

If these conceptions should prove tenable, we must then come to regard transmissible autolysis merely as a normal reaction carried to a "pathological" extreme, as has been held from the beginning by Bordet and Ciuca.[62]. In such a situation we should expect to be confronted in nature by lytic phenomena of such diversity and overlapping grades that no arbitrary line could be drawn between transmissible autolysis and certain other autolytic manifestations of less acute nature; and this, I believe, is the exact situation that I have shown to obtain in the bacterial

world today. I therefore regard the suggestion justifiable that active microbic dissociation and the phenomenon of the bacteriophage may represent merely two different stages in a single phase of normal reproductive and physiologic behavior which exists for a purpose presumably adaptive. Obviously, however, even to be convinced of the truth of this hypothesis, is still to be far from solving in its entirety the mystery of the bacteriophage; but it may well have the advantage of showing us in what direction we must look for the final solution.

And, finally, if for a moment we abandon ourselves to speculation, though not perhaps without some evidence in fact—it may eventually be demonstrated, not that a foreign filtrable virus gives rise to dissociation and to autolysis in the d'Herelle sense; but, on the contrary, that the fundamental physiologic reaction, of which both microbic dissociation and transmissible autolysis are only different modes of expression, gives rise to the filtrable virus.

ADDENDUM

In reviewing the final proof of this review, I am impressed with several important and quite unintentional omissions. Among these, one of unusual interest relates to the subject of "receptor analysis," developed especially at the hands of Felix[517] and Felix and Olitzki.[518] If, however, the interested reader will substitute the term, S antigen (labile), for their "large-flaking antigen" (or serums), and the term, O antigen (stable), for their "small-flaking antigen" (or serums), much of this highly important investigation on bactericidal serum action and qualitative receptor analysis may be brought into relation with certain aspects of the dissociation problem as presented particularly in sections 9 and 10 of the present work. With the possible exception of the older work on anthrax bacillus, I know of no other study dealing with the immunologic significance of cultures (or antigens) of the intermediate or O type, as opposed to the R forms. The latter were clearly not involved in the experiments of Felix and Olitzki. Even with this lack, however, their work is a singularly clear example of the vast significance of microbic dissociation for outstanding problems in serology and immunity.

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PLATE 1
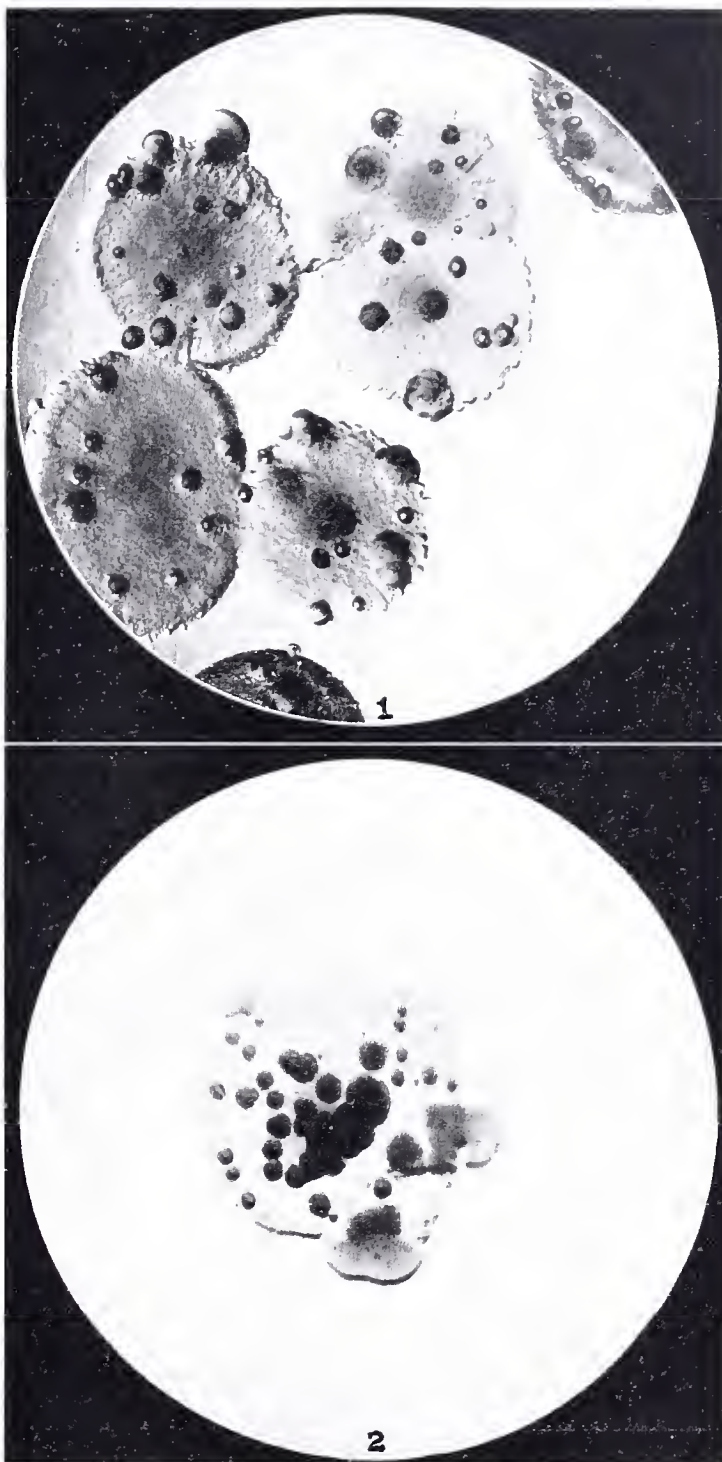
Fig. 1.—Vibro cholerae. Fourteen day old "helle" (S) colonies showing large surface secondary or daughter colonies. From Eisenberg,[150] magnification not given.

Fig. 2.—B. typhosus. Four day old colony on rhamnose agar, showing surface secondary colonies. From Reiner Müller:[358] Taf. II, fig. 3; × 5.
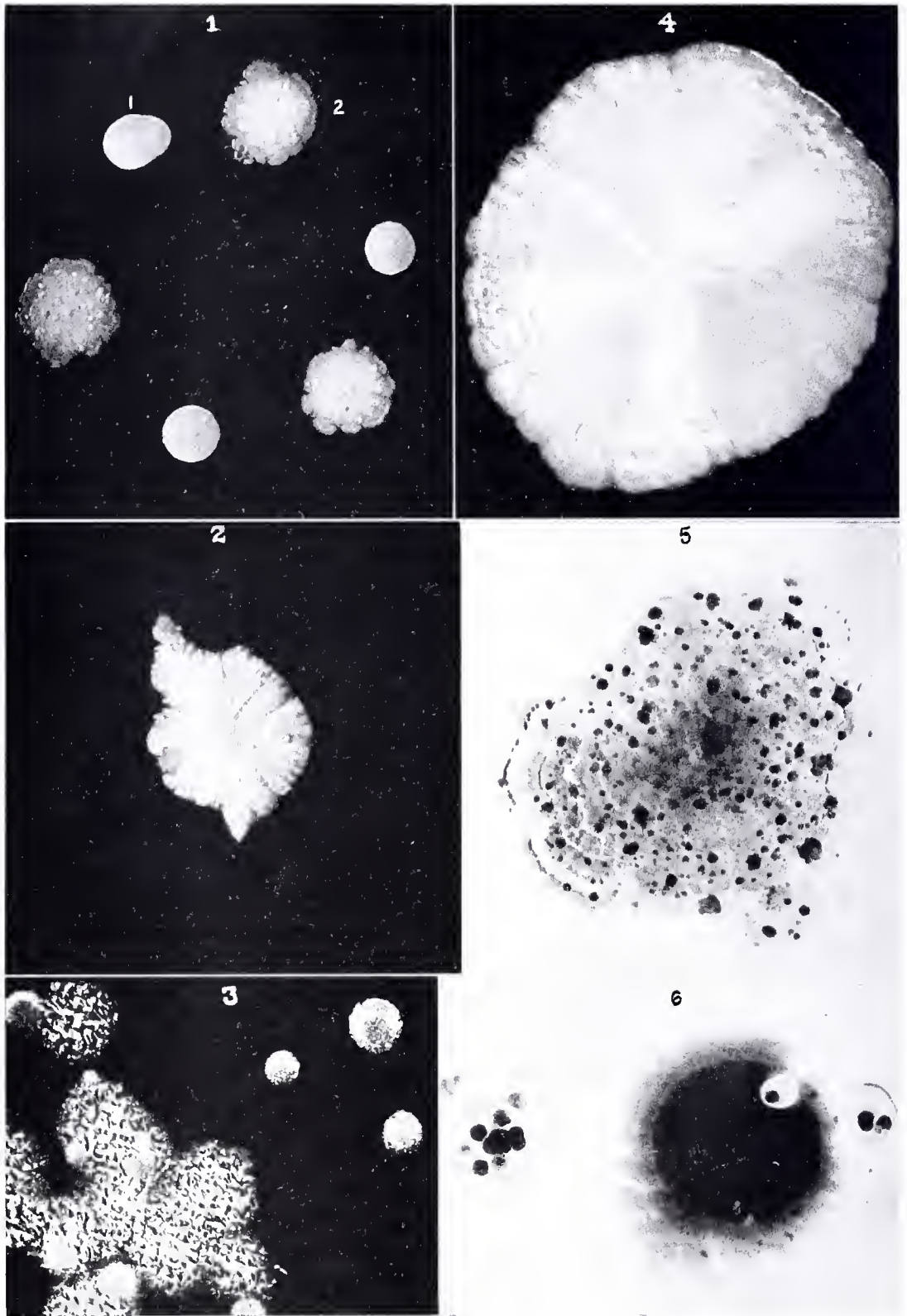
PLATE 2

Fig. 1.—Meningococcus. Alternate colonies of types II and III on deep "trypagar" plate. Shows difference between the restricted and the spreading growth. From Atkin:[21] Plate I, fig. 3; magnification not stated.

Fig. 2.—Meningococcus. Mature colony on deep "trypagar" plate. Shows irregular shape and "papillae" (daughter colonies). From Atkin:[21] Plate I, fig. 1; magnification not stated.

Fig. 3.—B. anthracis. Large, translucent typical Medusa-head (R) colonies and small, white, compact "atypical" (S) colonies. From Wagner:[479] Taf. II, fig. 20; × 10.

Fig. 4.—Gonococcus. Type II colony four weeks old on deep "trypagar" plate. Shows circular form, dense growth and absence of "papillae" or daughter colonies. The corrugated outer border does not represent papillae but is the effect of optical conditions. From Atkin:[22] Plate I, fig. 3; reflected light. × 4.

Fig. 5.—Gonococcus. Type I colony four weeks old on deep "trypagar" plate. Shows irregular shape, opaque papillae (daughter colonies) and transparent intervening area. Zonal effect shown on right-hand border. From Atkin:[22] Plate I, fig. 1; transmitted light, × 4.

Fig. 6.—B. typhosus. One "mutated" and three "mutating" colonies on rhamnose agar plate. The latter show daughter colonies. The large colony shows a regeneration fringe. From Reiner Müller:[355] Taf. I, fig. 2; × 5.

PLATE 3
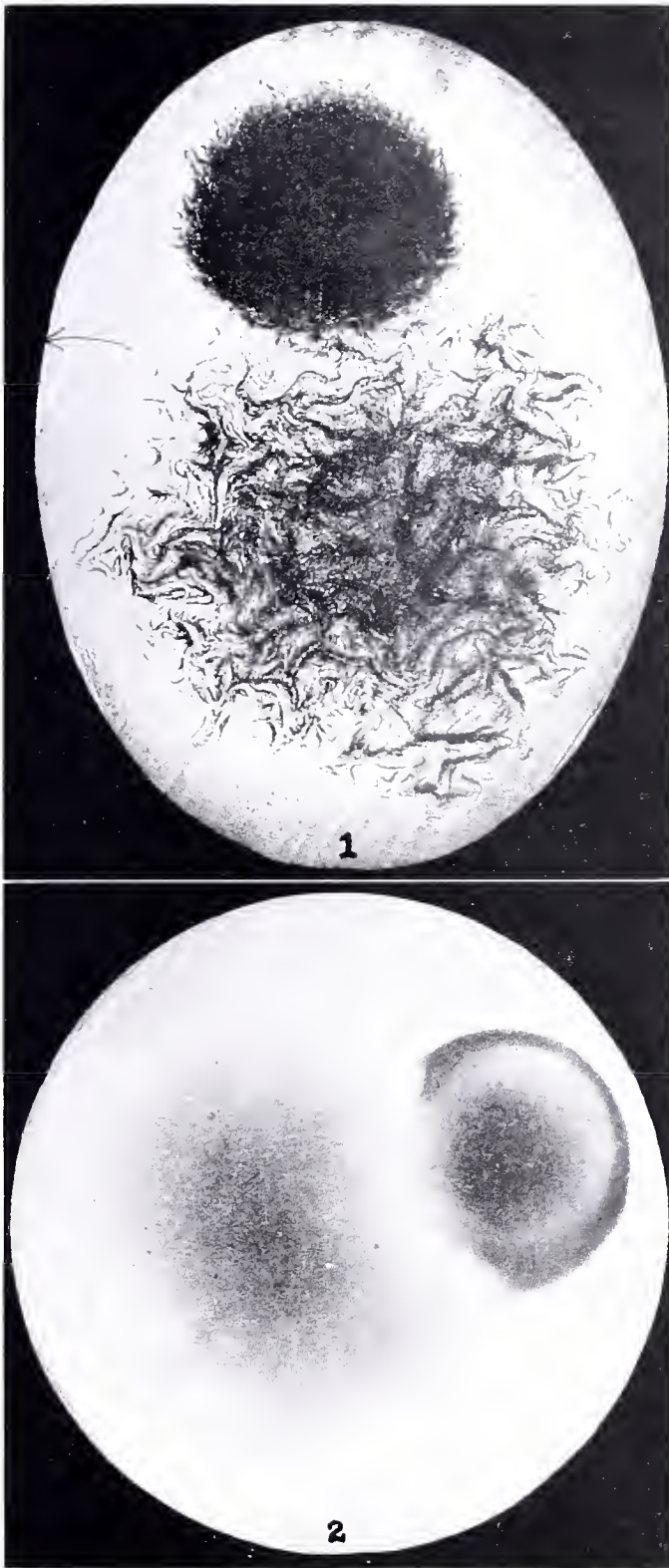
Fig. 1.—B. anthracis. Upper colony: Round, compact, opaque, whitish and "smooth," showing the "Krauskopf" appearance at border. Lower colony: Irregular, spreading, translucent, rough; showing typical anthrax, Medusa-head type. These two colonies are the same as the two colonies shown in the upper left corner of fig. 3 of plate 2. From Wagner:[176] Taf. II, fig. 22; transmitted light, × 50.

Fig. 2.—Vibrio cholerae. "Helle" (S) and dark, granular (O?) colonies, three days old. The dark colony contained many deep secondaries which do not appear in the photograph. From Eisenberg:[150] Taf. I, fig. 5; magnification not stated.
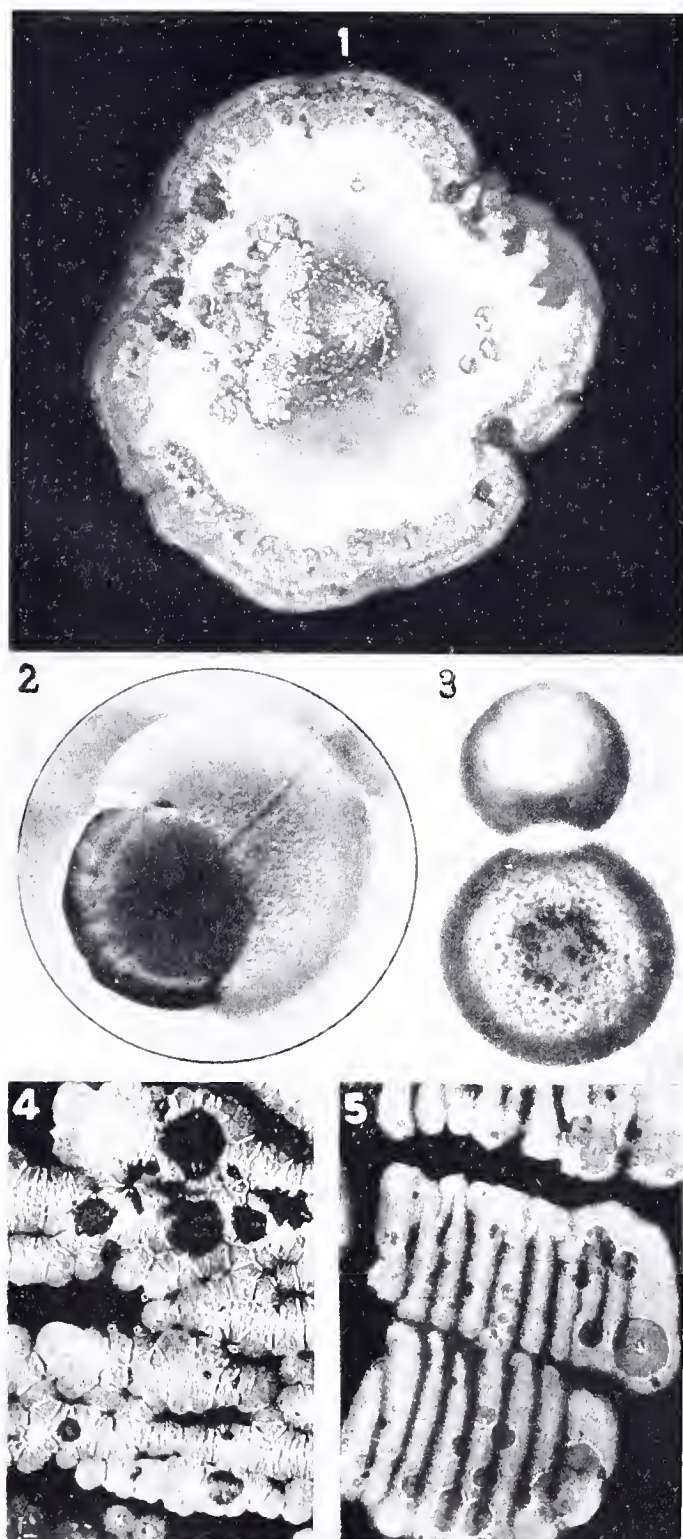
PLATE 4

Fig. 1. B. pyocyaneus. Lytic (and lysogenic) colony seven days old on beef-infusion-agar. Showing mass lysis, lytic pockets and marginal lysis well under way. For earlier stage of same colony see plate 5, fig. 2. From Hadley:[224] Plate II, fig. 7; reflected light, × 1.0.

Fig. 2.—Vibrio cholerae. "Helle" (S) (larger) and "dunkel" (O?) (smaller) colonies five days old on cholera-agar plates. From Eisenberg:[150] Taf. I, fig. 1; magnification not stated.

Fig. 3.—Vibrio cholerae. "Helle" (upper) and transitional (lower) colonies one day old. The latter shows the lysing central area. From Eisenberg:[150] Taf. I, fig. 2; magnification not stated.

Fig. 4.—Monilia culture from throat. Streak of Monilia on agar, showing lysing areas in the background of culture. From Sonnenschien:[448] Taf. I, fig. 5; magnification not stated.

Fig. 5.—B. pyocyaneus. Streaks on agar showing lysing areas against background of culture. From Sonnenschien:[448] Taf. I, fig. 7; magnification not stated.
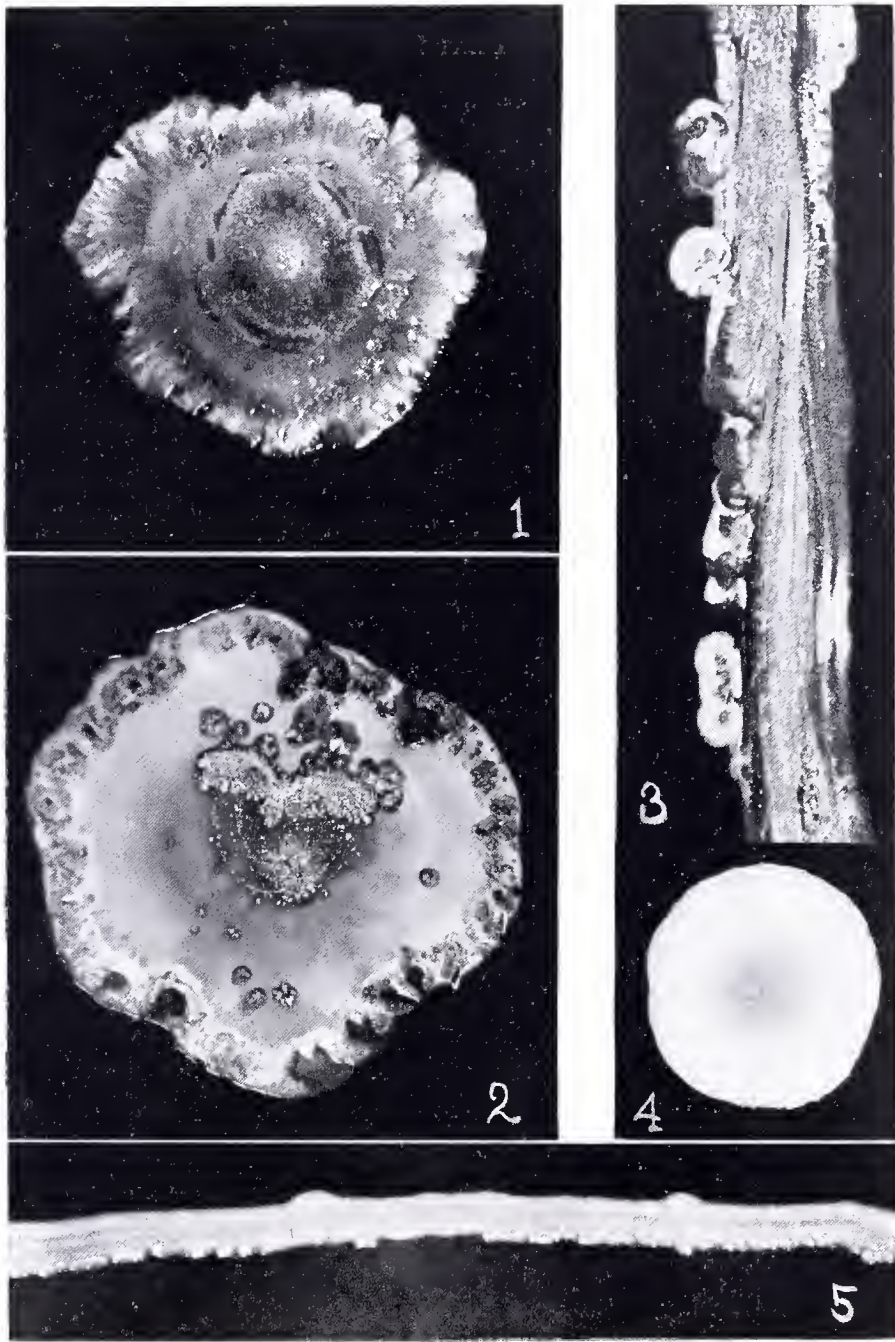
PLATE 5

Fig. 1.—B. pyocyaneus. Three days old lytic (and lysogenic) colony on beef-infusion-agar. Shows mass lysis and zonal areas of mass lysis. From Hadley:[224] Plate I, fig. 1; reflected light, × 1.2.

Fig. 2.—B. pyocyaneus. Four days old lytic (and lysogenic) colony on beef-infusion agar. Shows mass lysis, lytic pockets and marginal lysis. (For later stage of same colony see plate 4, fig. 1.) From Hadley:[224] Plate I, fig. 2; reflected light, × 1.0.

Fig. 3.—B. pyocyaneus. Streak growth of lytic culture five days old on beef-infusion-agar. Shows marginal lysis and erosions; also the proliferative growth as compared with fig. 5. From Hadley:[224] Plate I, fig. 3; reflected light, × 1.2.

Fig. 4.—B. pyocyaneus. R type colony on beef-infusion-agar, four days old. Shows smooth (unusual) surface. From Hadley:[224] Plate I, fig. 4; reflected light, × 1.0.

Fig. 5.—B. pyocyaneus. Five day old streak of R growth on beef-infusion-agar. Shows even growth free from lytic changes. Compare in extent of growth with fig. 3. From Hadley:[224] Plate I, fig. 5; reflected light, × 1.2.

PLATE 6
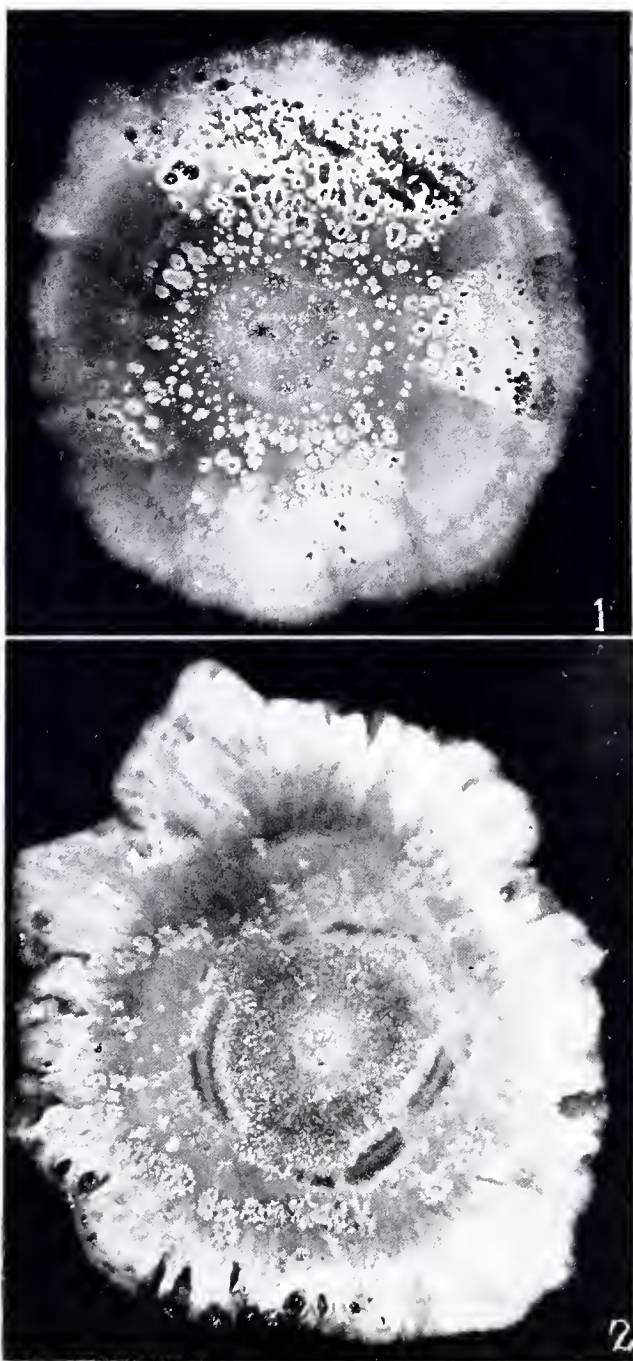
Fig. 1.—B. pyocyaneus. Lytic colony on infusion agar, 8 days old; showing radial and concentric zones of lysis, and the ringed lytic pockets and crystalline deposits. × 1.0. Hadley:[224] Plate 3, fig. 11.

Fig. 2.—B. pyocyaneus. Lytic colony on infusion agar, 8 days old; showing zonal areas of lysis and many lytic pockets. × 1.2. Hadley:[224] Plate 3, fig. 12.

A CASE OF PATHOGENICITY OF BACILLUS SUBTILIS

W. J. Bais

From Amsterdam, Holland

Cases of apparent pathogenicity of Bacillus subtilis are rare. Apart from their own case, Sweany and Pinner [1] found only one case in the literature of general disturbances caused by B. subtilis, i. e., a case of sepsis and pneumonia, described by Kelemen.[2] Otherwise pathogenic action of B. subtilis in man has always been found to be of a local character (eye lesions). This state of affairs justifies a short description of the following case which was observed by me in 1915 on Sumatra (D. E. I.).

A Javanese coolie, approximately 25 years of age, is admitted to the hospital on June 9, complaining of continual fever with severe chills and a painful cough since about 4 days. The patient is seriously ill, his right lung shows the physical signs of disseminated areas of infiltration, the left lung is normal; there is no expectoration. The spleen is palpable. No malaria parasites are found in the blood. The hemoglobin percentage is 50 (Tallquist), Pirquet's test is negative. The temperature is in the afternoon 41 C.; after a preliminary drop to 37 in the morning of the 10th it rises again to 40 the same afternoon and remains between 39.5 and 41 C. till the 16th; from that time till death it was 38-39.5 C.

June 11 the right lung shows signs of a cavity of about the size of an orange (tympany, amphoric breathing sounds, coarse gurgling rales) in front downward from about one inch under the collar bone. The rest of the right lung shows infiltration and bronchitic murmurs. The urine contains a trace of albumen, otherwise nothing abnormal, the diazo test is negative. The leukocyte count is 35,500; differential count: basophiles 0, eosinophiles 0, young polymorphonuclear neutrophiles 3, neutrophiles with rod shaped nucleus 28, with segmented nucleus 61, lymphocytes 4, large mononuclears 4.

Two cc. of blood is taken from the cubital vein and a plate culture made with ordinary nutrition agar.

June 12 the patient's condition is the same. The agar plate shows a few black colonies, some of which (the superficial ones) are surrounded by a hemolytic area.

The cultures are transferred to other medium. The strain grows well in ordinary broth, on agar slants slowly in the beginning, but much more quickly after another transfer, forming a dry coating adherent to

[1] J. Infect. Dis., 1925, 37, p. 340.
[2] Gyogyaszat, 1924, 64, pp. 512, 531, 548, 561; Abstr. Zentralbl. f. d. ges. Tuberkfg., 1925, 24, p. 22.

the medium. It is a fairly large bacillus, keeping the gram stain and producing central spores. It has a slow type of motility, making more or less wagging movements. In gelatin it quickly causes liquefaction.

The first impression of the microscopic picture was that of the anthrax bacillus; the motility (much more marked after growth in broth) and the quick liquefaction of gelatin soon established the correct diagnosis: B. subtilis. The organism has frequently been confounded with B. anthracis.

June 15.—Symptoms of cavities are present practically over the whole of the right lung; the left lung remains free. The patient brings up putrid sputum with much difficulty.

As B. subtilis is a common source of contamination in bacteriological work the blood culture is repeated with exactly the same results as before. It may be added that none of my other cultures during this period showed signs of infestation with B. subtilis.

The next few days the patient's condition gets worse and in the early morning of June 22 he dies.

A postmostem examination was made the same day. The record sheet mentions, that extensive cavitation was found in the right lung, with foul smelling contents; the left lung showed only a few scattered areas of light infiltration; there were no signs of tuberculosis. (The detailed postmortem report is lost.)

Scrapings from the wall of the cavities and from a section of the spleen were inoculated on agar and broth. The former cultures yielded various colonies, amongst which B. subtilis could not be detected; the latter remained sterile.

In smears taken from the walls of the cavity several specimens of microorganisms were seen, probably including B. subtilis, but no acidfast bacilli were found.

The very acute course of the disease in a previously healthy individual and the absence of tuberculous lesions elsewhere a priori made tuberculosis in this case improbable.

It is to be regretted that at the time, being far from the resources of literature, the significance of this case was not adequately recognized and that thereby neither the cultures have been subjected to further study (agglutination, etc.), nor animal experiments have been made with it. But the facts that B. subtilis was cultivated twice from the patient's blood, and that the strains in their behavior (hemolytic action and growth on agar) closely resembled those found by Sweany and Pinner, are suggestive of their pathogenic significance in this case. The failure to obtain the bacillus in culture from the affected lung may be explained by the fact that the medium used by me for this purpose contained no blood,

the addition of which according to Sweany and Pinner is necessary, at least to obtain successful primary cultures.

It is remarkable that in two of the three cases of general pathogenic action of B. subtilis the infection has apparently entered the system by the respiratory way, whilst in Kelemen's case the infection, though acquired in another way (accidentally in a surgical operation), also localized itself in the lung. This predilection for the respiratory organs is a noteworthy feature in connection with the pronounced aerobic habits of the bacillus.

It is still obscure what influences may render the bacillus pathogenic, though it is quite plausible, in view of the results of animal experiments (Charrin and De Nitis, Kelemen[2]), that it may be caused by repeated passing through animals, from which the infection might be acquired by man. Kelemen appears to be of a similar opinion. Yet nothing is known so far about such contact.

As in Kelemen's case, and more than in Sweany and Pinner's case in which the secondary character of the B. subtilis infection was beyond doubt, the features of the case reported by me suggest a possible pathogenicity of this bacillus for man. Anyhow it supports their incitation to pay more attention to B. subtilis than it usually receives from the human pathologists.

SUMMARY

A case of gangrene of the lungs with the isolation of B. subtilis in cultures from the blood is reported.

THE IMMUNOLOGIC BEHAVIOR OF MUCOIDS

JULIAN H. LEWIS AND H. GIDEON WELLS

From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago

As the mucoids are not coagulated by heat and are probably widely distributed in the animal body, they offer a complicating factor in immunologic studies of heated antigens which has ordinarily not been taken into account. Constituting about 5% of egg white proteins and from 0.5 to 1% of the serum proteins [1] they are sufficient in amount to be of significance in immunologic reactions with these materials, and especially so when the coagulable proteins have been more or less removed or altered by heat. Since little is known concerning their antigenic capacity and properties we have sought to extend our knowledge of their immunologic behavior.

As far as we can learn the first immunologic studies that have been made with ovomucoid are the anaphylaxis experiments reported by one of us in 1911.[2] In these it was shown that ovomucoid was capable of producing characteristic anaphylaxis reactions, sometimes but not often fatal when the intraperitoneal route of injection was used. It was also found that the ovomucoid of hen's eggs is immunologically distinct from crystallized egg albumin, purified egg globulin, and ovovitellin of hen's eggs.

The immunologic reactions of other glucoproteins have been studied in this laboratory by Elliott,[3] who isolated ox submaxillary mucin, swine stomach mucin and ox tendon "mucin," the last of which is considered to be less closely related to the mucins than to the mucoids or the chondroproteins. This tendon mucoid was found to give typical anaphylaxis reactions with itself as antigen, and to be distinct from the two gastric mucins. Likewise it produced precipitins which reacted much more strongly with itself than with either of the gastric mucins, although enough reactions were obtained with these mucins to indicate the existence of some chemical relationship between the three glucoproteins, even when from different species of animals. Similar results were obtained with the complement fixation reaction.

[1] Bywater: Biochem. Ztschr., 1909, 16, p. 322.
[2] Wells. J. Infect. Dis., 1911, 9, p. 147.
[3] Elliott: J. Infect. Dis., 1914, 15, p. 501.

Goodner [4] has tested certain glucoproteins by the precipitin reaction, finding chondromucoid, tendon mucoid, trachea mucoid and submaxillary mucin of beef to be immunologically distinct from each other and from beef serum and from mucin from hog stomachs. All the above proteins were distinct from ovomucin and ovomucoid of hen's eggs, although the two latter preparations were not qualitatively distinct from each other according to the precipitin tests. It was found to be very difficult to produce precipitins with these glucoproteins, and only occasionally were active antiserums obtained. No precipitin sera could be obtained by immunizing with tendonmucoid or beef trachea mucoid. The most active serums with the mammalian glucoproteins gave reactions only in dilutions of 50 to 200, whereas ovomucin gave a precipitin reacting at a serum dilution of 1 in 1,600.

An interesting case is reported by Schloss [5] of a boy, hypersensitive to eggs, who was found to give a stronger cutaneous reaction to ovomucoid than to any other of the proteins of eggs, and whose serum rendered guinea-pigs passively sensitive to ovomucoid. Feeding ovomucoid in gradually increasing doses removed the hypersensitivity of the patient.

We can find no reports of investigation of the antigenic properties of seromucoid, nor any evidence that it has been taken into consideration in investigations dealing with the immunologic properties of heated serum in which it must play a very important part.

EXPERIMENTS WITH OVOMUCOIDS

Ovomucoids were prepared from the eggs of hen, goose, turkey, duck, guinea hen, ostrich and turtle,* all of which were found to contain a protein fraction not coagulable by boiling and giving reactions corresponding with those of the ovomucoid of hen's eggs. Mörner found ovomucoid in the eggs of all of 96 species of birds.[6]

Anaphylaxis tests indicated that these ovomucoids are not very active as antigens, as had been observed previously in experiments with hen ovomucoid.[2] In that series of experiments, 35 guinea-pigs were sensitized with five different prepa-

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* Most of these preparations were made by Dr. Mary E. Maver, according to the following method: The eggs were beaten and diluted with 3 volumes of water. This mixture was added slowly to 5 volumes of boiling water. Enough dilute acetic acid was added to make the reaction slightly acid. After the mixture had boiled for about 5 minutes it was filtered hot. The filtrate was evaporated until a flocculent precipitate began to appear. The solution was filtered and the ovomucoid precipitated by adding 4 volumes of 95% alcohol. The precipitated ovomucoid was purified by redissolving in a minimum amount of water and reprecipitating with alcohol. This procedure was repeated 3 or 4 times, and the ovomucoid was finally dried with alcohol and ether. The ovomucoids dissolve readily in water and are not coagulated by boiling or by acetic acid. They give a strong Fehling's reaction. They also show uncombined sulphur. The color reactions for tryptophan are positive. Millon's reaction is positive.

[4] J. Infect. Dis., 1925, 37, p. 285.
[5] Am. J. Dis. Child., 1912, 3, p. 341.
[6] Ztschr. f. physiol. Chem., 1912, 80, p. 430.

rations of hen ovomucoid and given reinjections intraperitoneally with the corre-
sponding protein, with the result that 4 fatal, 9 severe, 8 moderate, 9 slight and
5 negative or doubtful reactions were obtained.

When we sensitized guinea-pigs with hen ovomucoid and reinjected with 50 to
100 mg. of guinea-hen, ostrich, duck or goose ovomucoid by the intraperitoneal
route * we failed to obtain any reactions strong enough to be considered as
unquestionably positive, although some doubtful symptoms were obtained. In a
few instances the heterologous protein was injected into the heart in 10 mg. doses
without producing reactions. After failing to react to the heterologous proteins
the animals were found to be more or less reactive to intracardiac injections of
hen ovomucoid, although the reactions were less severe than they usually are with
hen ovomucoid given by this route.

Similar results were obtained in crossed reactions in which guinea-pigs were
sensitized with one of the other ovomucoids (goose, ostrich, duck, turkey and guinea-
hen) and given the second or intoxicating dose with a heterologous ovomucoid.
Even when the second dose was given by the intracardiac route the reaction was
usually not serious, except in two cases in which guinea-hen ovomucoid gave
fatal reactions in animals sensitized, one with ostrich and the other with goose
ovomucoid. When the second injection was made with the homologous protein
by the intracardiac route, fatal anaphylactic shock was usually obtained, whether
or not there had been an intermediate injection of some heterologous ovomucoid.
On the other hand, when large doses (50 to 100 mg.) of the heterologous proteins
were given into the peritoneal cavity they commonly produced at least a slight or
doubtful anaphylaxis reaction, never severe, but such doses often rendered the
animal refractory to the homologous protein, even by the intracardiac route.

These results suggest a relationship but not an identity between the
several ovomucoids, but the antigenic activity of these proteins is so low
that the simple anaphylaxis test does not serve to determine the degree
of this relationship. Therefore, experiments were tried by other methods.

Complement Fixation Tests.—Rabbits were immunized with ovomucoids and
their serum tested by complement fixation reaction with various mucoids. Anti-
body production was rather slow with these antigens as compared with such anti-
gens as horse serum and egg white. Several "courses" of immunzation were given
in order to produce antiserums potent enough to use. While such antiserums
gave satisfactory complement fixation reactions with a sensitive system, the pre-
cipitin reactions with the ovomucoids were always very weak, as was also observed
by Goodner.[4]

The results of complement fixation tests are given in tables 1 and 2.
These show that when the immunization is not carried too far (table 1)
the degree of specificity shown by the immune serum is much greater
than when the immunization is carried further (table 2). Table 1 shows
a close relationship between duck and goose ovomucoid, but the other
mucoids do not give reactions with this antiserum for goose ovomucoid.
Both tables show a close relationship between hen and guinea fowl ovo-

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* From five to ten experiments were carried out with each combination of injections
mentioned. The publication of full protocols seems unnecessary in view of the character of
the results.

mucoid, although there is a quantitative difference that is sufficient to indicate that these two ovomucoids are distinctly different. When the immunization against hen ovomucoid is pushed until a very strong antiserum is obtained, some reaction is given by the higher concentrations of all the ovomucoids tried (table 2), but the quantitative

TABLE 1

COMPLEMENT FIXATION TESTS WITH ANTISERUMS FOR GOOSE AND HEN OVOMUCOIDS

	Antiserums				
	Goose Ovomucoid, Mg. Antigen	Hen Ovomucoid,* Mg. Antigen			Normal Serum, Mg. Antigen
Antigens	0.5	0.5	1.0	5.0	5.0
Ovomucoids					
Goose......................	++++	0	0	0	0
Duck.......................	++++	0	0	0	0
Hen........................	0	++++	++++	++++	0
Guinea fowl.................	0	+	++	+++	0
Ostrich.....................	0	0	0	0	0
Turkey.....................	0	0	0	+	0
Seromucoids					
Beef.......................	0	0	0	±	0
Sheep......................	0	0	0	±	0
Muscle mucoid, beef.............	0	0	0	+	0

TABLE 2

Ovomucoid Antigens	Hen Ovomucoid Antiserum,* Mg. Antigen					Normal Serum, Mg. Antigen
	0.005	0.025	0.05	0.5	5.0	5.0
Goose..............	0	0	0	++++	++++	0
Duck...............	0	0	++++	++++	++++	0
Hen................	++++	++++	++++	++++	++++	0
Guinea fowl.......	0	++	++++	++++	++++	0
Ostrich............	0	0	0	0	++++	0
Turkey............	0	0	++++	++++	++++	0

* The hen ovomucoid antiserum used in table 2 is from the rabbit producing the serum in table 1, after the rabbit had been further immunized.

differences still show that hen ovomucoid is in some respect different from all the others, and most like the guinea fowl ovomucoid.

Table 3 shows the result of complement fixation tests with another preparation of antiserum for goose ovomucoid, which again establishes the close relationship of goose and duck ovomucoids, and their difference from all the other ovomucoids.

Table 4 shows the results of tests with a very potent antiserum for turkey ovomucoid. This serum was so active that it gave strong complement fixation reactions with all the other ovomucoids, and even some reactions with strong concentrations of the seromucoids from the blood of sheep and dogs. Quantitatively the turkey ovomucoid is distinctly different from the others, but the difference is relatively slight.

TABLE 3

COMPLEMENT FIXATION TESTS WITH ANTISERUM FOR GOOSE OVOMUCOID

Ovomucoids	Mg. Antigen							Serum Control	Antigen Control
	5	1	0.5	0.1	0.05	0.01	0.005		
Guinea-hen...	0	0	0	0	0	0	0	0	0
Hen..........	++	0	0	0	0	0	0	0	0
Goose........	++++	++++	++++	+++	0	0	0	0	0
Duck..........	++++	++++	++++	++	0	0	0	0	0
Ostrich.......	+	0	0	0	0	0	0	0	0
Turkey.......	0	0	0	0	0	0	0	0	0
Sheep...... ..	++++	0	0	0	0	0	0	0	0
Dog...........	++++	0	0	0	0	0	0	0	0

TABLE 4

COMPLEMENT FIXATION TESTS WITH STRONG ANTISERUM FOR TURKEY OVOMUCOID

Ovo-mucoids	Mg. Antigen										Serum Control	Antigen Control
	5	1	0.5	0.1	0.05	0.01	0.005	0.0025	0.0005	0.00025		
Guinea-hen....	++++	++++	++++	++++	+++	++++	++++	++++	+	0	0	0
Hen.....	++++	++++	+++	++	++	++++	++++	+	0	0	0	0
Goose...	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0	0
Duck....	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0	0
Ostrich..	++++	+	0?	0?	0?	+++	+++	+	0	0	0	0
Turkey..	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0	0
Turtle...	++++	++++	++++	+++	0	0	0	0	0	0	0	0
Sheep....	++++	+	0	0	0	0	0	0	0	0	0	0
Dog.....	++++	++	+	0	0	0	0	0	0	0	0	0

Uterine Strip Anaphylaxis Reactions.—This has been in our hands the most satisfactory method for detecting differences between closely related proteins, because of the readiness with which saturation to the heterologous antigen is accomplished and the sharpness of the reactions. Small virgin female guinea-pigs were sensitized with 10 mg. of the various ovomucoids, and after an incubation period of 14 to 21 days, uterine horn preparations were tested in the Dale apparatus.

The following summary gives the results obtained when a sensitized uterine strip was tested with a series of different proteins in the order stated:

1. Sensitized with ostrich ovomucoid
   reacted with duck, desensitized
   reacted to hen, desensitized
   reacted to guinea hen
   reacted to ostrich

2. Sensitized with ostrich
   reacted with turkey, desensitized
   reacted with guinea hen
   reacted with hen
   reacted with ostrich

3. Sensitized with turkey ovomucoid
   reacted to hen, desensitized
   reacted to guinea hen
   slight reaction to duck
   reacted to turkey

4. Sensitized with turkey ovomucoid
   reacted to guinea hen, desensitized
   reacted to goose, desensitized
   slight reaction to ostrich
   reacted to turkey

5. Sensitized to guinea hen
   reacted to duck
   no reaction to hen
   reacted to guinea hen

6. Sensitized with guinea hen
   reacted to turkey
   no reaction to goose
   no reaction to hen
   reacted to guinea hen

7. Sensitized with guinea hen
   reacted with hen, desensitized
   small reaction with duck
   reacted to guinea hen

8. Sensitized with goose
   reacted with guinea hen
   no reaction with hen

   no reaction with ostrich
   reaction with duck
   no reaction with goose

9. Sensitized with goose
   reacted with ostrich
   reacted with guinea hen
   no reaction with hen
   no reaction with turkey
   no reaction with duck
   reaction with goose

10. Sensitized with duck
    reacted with goose, desensitized
    small reaction with duck

11. Sensitized with turtle
    no reaction with ostrich
    reacted with turtle

12. Sensitized with turtle
    no reaction with guinea hen
    no reaction with ostrich
    reacted with turtle

    2nd horn
    no reaction with duck
    reacted with turtle

13. Sensitized with turtle
    no reaction with dog seromucoid
    no reaction with hen
    reacted with turtle

    2nd horn
    no reaction with hen
    no reaction with sheep seromucoid
    no reaction with turkey
    no reaction with goose
    no reaction with ostrich
    no reaction with guinea hen
    reacted with turtle

In general these tests showed the close relationship between the various avian ovomucoids, corroborating the results of the anaphylaxis shock and complement fixation tests. In nearly all cases the uterine strip sensitized to any one of the ovomucoids would give strong reactions with any other ovomucoid tried. If then tested with some other heterologous ovomucoid it might or might not give a reaction, but even when by repeating the addition of heterologous antigens the uterine strip was desensitized to this heterologous antigen, it still retained a marked sensitivity to the homologous antigen, i. e., the one to which it had been originally sensitized. This is illustrated in the following curves, which are a few of the experiments given in the preceding summary. This behavior of the sensitized uterine strip establishes the fact that these various ovomucoid preparations are similar to one another, but despite the similarity they are demonstrably different from one another. Corresponding with the complement fixation tests, the duck and goose ovomucoids are distinctly more closely related than any of the others, and
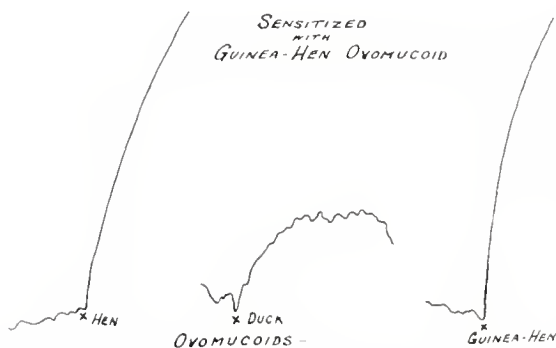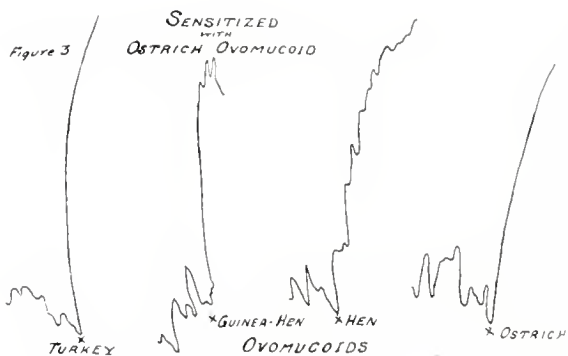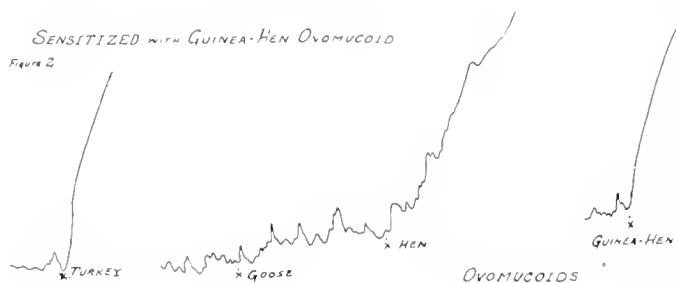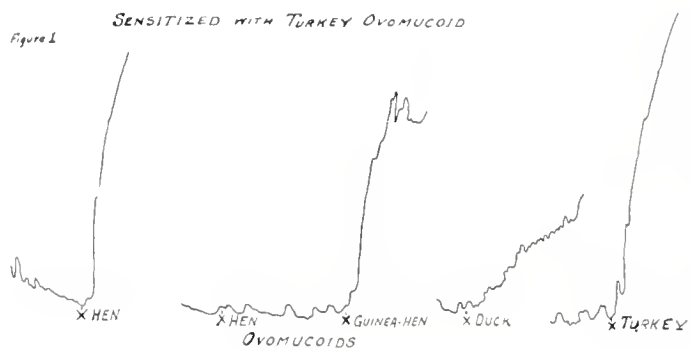
Figs. 1, 2, 3 and 4.—Tracings of reactions of guinea-pig (sensitized as indicated) uterine muscle strips to different ovomucoids.

are perhaps identical, since desensitization with one renders the animal refractory to the other (figs. 5 and 6).

The turtle ovomucoid seems to be distinct from the ovomucoids of the birds, since uterine strips sensitized to turtle ovomucoid gave no reactions with the ovomucoids from birds' eggs (exper. 11, 12, 13). This agrees with the observation that the highly active antiserum for turkey ovomucoid (table 4) gave less complement fixation with turtle ovomucoid than with any of the bird ovomucoids.
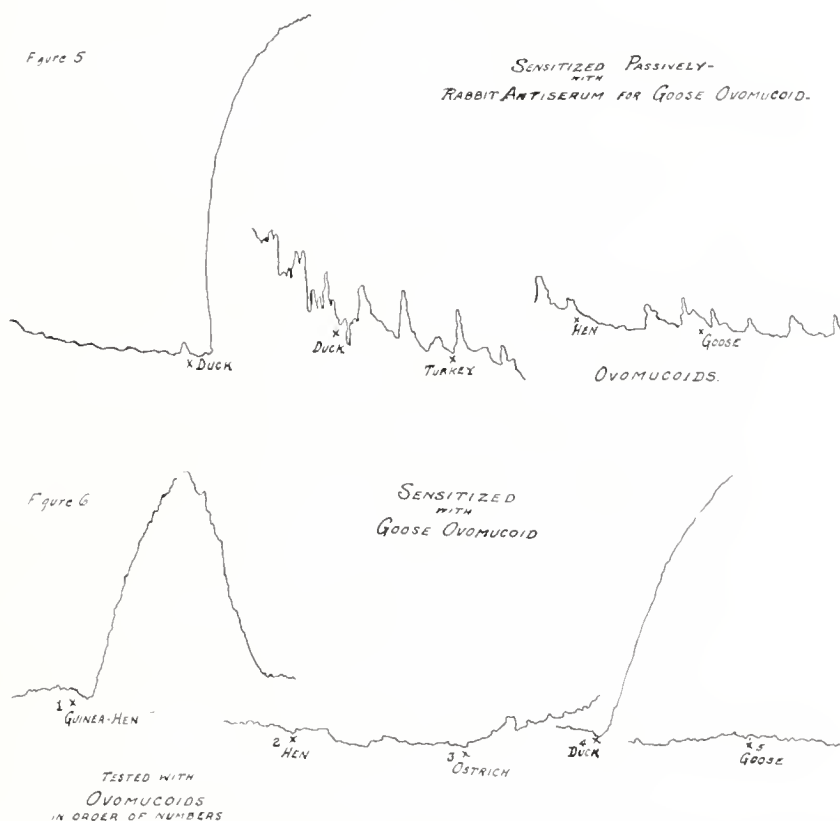
Figs. 5 and 6.—Tracings of reactions of uterine strips from guinea-pigs sensitized to goose ovomucoid, both actively and passively, showing apparent identity of duck and goose ovomucoids.

EXPERIMENTS WITH SEROMUCOIDS

Seromucoid preparations were made from sheep blood and beef blood by Miss Maver, following approximately the methods used by Bywater.[1]

The blood was treated with 1% sodium citrate, diluted with four volumes of water, and made slightly acid with sulphuric acid. It was heated to boiling with steam and then boiled for about five minutes. The precipitate was filtered hot and washed by boiling with two volumes of water. The collected filtrates were evaporated to a small volume and precipitated with three volumes of alcohol. The

crude seromucoid was dissolved in a minimum amount of water and reprecipitated with alcohol several times, and then dialyzed. The seromucoid was finally precipitated with alcohol and dried with alcohol and ether.

The seromucoid is soluble in hot water and is not precipitated on boiling. It gives negative reactions for tryptophan and indol. When treated with KOH and lead acetate no lead sulfide is formed. It gives the reactions for glucoproteins in general.

In anaphylaxis experiments the beef seromucoid was not very active, producing only slight reactions when 50 mg. doses were injected intraperitoneally into sensitized guinea-pigs, or with 5-10 mg. intracardiac doses, but fatal results were sometimes obtained with 20 mg. doses into the heart. It did not sensitize to normal beef serum.

The sheep seromucoid preparation was much more active. Fifty milligram doses intraperitoneally produced slight typical reactions only,

. TABLE 5

Complement Fixation Reactions with Antiserum for Antisheep Seromucoid

Antigens	Mg. Antigen							Serum Control	Antigen Control
	5	1	0.5	0.1	0.05	0.01	0.005		
Ovomucoids									
Guinea-hen.	++	0	0	0	0	0	0	0	0
Hen.........	+++	0	0	0	0	0	0	0	0
Goose.......	+	0	0	0	0	0	0	0	0
Duck........	++++	++	±	0	0	0	0	0	0
Ostrich.....	+++	0	0	0	0	0	0	0	0
Turkey.....	+	0	0	0	0	0	0	0	0
Seromucoids									
Sheep.......	++++	++++	++++	++++	++++	++	0	0	0
Dog.........	++++	++	0	0	0	0	0	0	0

but protected the animal against 10 mg. injections into the heart, which dose was usually fatal by the cardiac route.

Guinea-pigs sensitized with sheep seromucoid gave slight reactions on reinjection with 50 mg. of beef seromucoid, but were not protected against sheep seromucoid, which gave fatal reactions on subsequent intracardiac injections. Guinea-pigs sensitized with sheep seromucoid gave very slight reactions with whole sheep serum.

A preparation was made by treating ground up beef in the same way as the serum is treated in making seromucoid, but the resulting product was totally inert in anaphylaxis experiments. It did not sensitize to itself or to beef serum.

Complement fixation experiments with the serum of a rabbit immunized to sheep seromucoid showed a considerable specificity, even when compared with seromucoid from dog's blood (table 5). The ovomucoids reacted only in high concentrations. As shown in the tables giving the results of tests with the antiserums for goose and turkey ovomucoids,

these also failed to react with the sheep and dog seromucoid. Our preparation of beef seromucoid was found to be anticomplementary, and so could not be tested. Furthermore, it was so oxytoxic that it could not be used for uterine strip tests.

Uterine strip tests of the seromucoid from dogs and sheep blood, also showed them to be immunologically distinct from each other and from ovomucoid. The results of typical experiments are summarized as follows:

Sensitized with sheep seromucoid: No reaction to hen ovomucoid; reacted to sheep seromucoid. Second horn: Atypical reaction to dog seromucoid; reaction to sheep seromucoid.

Sensitized with sheep seromucoid: No reaction with dog sermucoid; reacted with beef seromucoid (oxytoxic); no reaction with hen ovomucoid; reacted with sheep seromucoid.

SUMMARY

Mucoids from eggs and from blood are antigenic, but less so than most other proteins of equal solubility. This may depend on the vigorous treatment required for their isolation.

Mucoids from the eggs of several species of birds show some immunologic relationship, but can generally be distinguished from each other by quantitative results or saturation experiments, especially by the uterine strip method of Schultz and Dale. Most closely related to each other are the ovomucoids from goose and duck eggs, which are perhaps identical. Hen and guinea-fowl ovomucoids are also closely related. Ovomucoid from turtle eggs is less closely related to the avian ovomucoids than these are to each other.

Seromucoids from sheep, beef and dog blood seem to be easily distinguishable from one another, and they show but little relationship to the ovomucoids.

IMMUNOLOGIC REACTIONS OF THE GLOBULINS FROM THE SEEDS OF LEGUMINOUS PLANTS

THE BIOLOGIC REACTIONS OF THE VEGETABLE PROTEINS, IX.

H. Gideon Wells, Julian H. Lewis

AND

D. Breese Jones

From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago, and the Protein Investigation Laboratory, U. S. Department of Agriculture, Washington, D. C.

Few investigations of the proteins of beans by immunologic methods have been recorded. Some observations have been made with crude extracts of beans, such as those of Bertarelli,[1] who immunized with Phaseolus vulgaris, pea, lentil, and vetch, tested the immune serums with extracts of various leguminous seeds, and found a distinct quantitative specificity, although not a qualitative specificity; e. g., an antivetch serum which gave precipitin reactions at dilutions of 1 to 3,000 with vetch extracts, reacted only at 1:100 to 1:300 with the other extracts. Relander[2] made a preliminary report corrobating Bertarelli's observations. Gasis[3] obtained a similar specificity with the fraction of bean proteins that was not coagulated by heat.

Raubitschek[4] found that antiserum for extracts of lentils and beans inhibits the hemagglutinins present in such extracts. He produced anaphylaxis reactions with these extracts, as also did Karasawa,[5] who used water extracts of rice, wheat, and "beans" (species not stated). Hiki[6] obtained non-specific precipitin reactions with antiserum against extracts of various beans, and also with the protein fraction obtained by saturating with ammonium sulfate.

The only experiments recorded in which isolated proteins from beans and other leguminous seeds were studied have been reprted in previous articles of this series.[7] The proteins investigated were the legumins from vetch (Vicia sativa), peas (Pisum sativum), lentils (Ervum lens), and the horse bean (Vicia faba); vicilin from peas; vignin from the cow-pea (Vignia sinensis s. catjang); glycinin from the soy bean (Soja hispida); phaseolin from the kidney bean (Phaseolus vulgaris) and from the Japanese adzuki bean (P. angularis); and the soluble, noncoagulable proteins, commonly and erroneously called "proteoses," obtained from peas, lentils, soy beans, and adzuki beans. In most of the experiments only the simple anaphylaxis reaction, with intraperitoneal injection of the proteins, was employed, a method that is relatively crude, yielding results that must be looked upon as essentially preliminary to investigations made by more accurate methods.

[1] Centralbl. f. Bakteriol., 2, O., 1904, 11, pp. 8, 45.
[2] Ibid., 1908, 20, p. 518.
[3] Berl. klin. Wchnschr., 1908, 45, p. 358.
[4] Wien. klin. Wchnschr., 1909, 22, p. 1752.
[5] Ztschr. f. Immuntätsforsch. u. exper. Therap., 1910, 5, p. 509.
[6] Sci. Rept. Gov't. Inst. Infect. Dis., Tokyo Imperial Univ., 1922, 1, p. 163.
[7] Wells and Osborne: J. Infect. Dis., 1911, 8, p. 66; 1914, 14, p. 377; 1915, 17, p. 259; 1916, 19, p. 183. Elsesser: ibid., 1916, 19, p. 655.

The net results of these studies were as follows: The various sorts of proteins prepared from these seeds possess the capacity to produce typical anaphylaxis reactions in guinea-pigs, as well as precipitin and Abderhalden "defensive ferment" reactions with the serum of immunized animals. In anaphylaxis tests the activity of the proteins largely depends on the solubility of the foreign protein in the body fluids of the animal, and hence particularly strong reactions are obtained with the highly soluble fraction present in these as in other seeds, which have usually been designated as "proteoses" because of their solubility. However, the more abundant proteins from these seeds are also fairly soluble in the body fluids and therefore are found to be as effective in producing anaphylaxis reactions as many other vegetable proteins. The principal protein, legumin, of pea, vetch, lentil and horse bean seems to be very similar if not identical in these four seeds, according to both chemical and immunological criteria. A preparation of vicilin of peas, a substance very difficult to separate from legumin, was found to be related to or contaminated with pea legumin, for crossed reactions were obtained with pea legumin and also with vetch legumin, although vetch contains no vicilin. Vignin, which, resembles legumin, but appears to be chemically distinguishable, seemed to be distinct from pea legumin, but as relationship to vetch legumin was indicated by some reactions, these results, being contradictory, are of doubtful value. Glycinin, the chief protein of the soy bean, was distinct from legumin and vicilin, but some crossed reactions were obtained with vignin. The phaseolins from adzuki beans and from kidney beans seemed to be immunologically distinct.

The "proteoses" from these leguminous seeds were found, like the corresponding proteins from other seeds, to be very active antigens, which indicates that they are not proteoses formed as cleavage products of larger protein molecules, since such proteoses are little if at all antigenic.[8] These leguminous "proteose" preparations were found to be entirely distinct from the other proteins of the same seeds, and from each other. Their chemical relationships have not been investigated.

Elsesser's studies[7] of these vegetable proteins by means of the Abderhalden reaction gave the same discordant results that were obtained by this method when other purified proteins were studied.

Because of the number and varied relationship between the plants of this group, and the fact that some of the seeds produce several different proteins which have been the subject of chemical study, they have furnished useful evidence concerning the dependence of immunologic specificity upon chemical composition. For example, the protein called legumin obtained from the seeds of the pea, vetch, lentil and horse bean shows no recognizable chemical differences when derived from the several different seeds, and preparations of legumin from these four seeds were found to give crossed anaphylaxis reactions as if they were all quite the same. On the other hand, when two chemically different proteins from the same species are tested against one another, e. g., the proteose and the legumin of the lentil, they give no crossed reactions, or but faint ones. Or when chemically different proteins from biologically closely related species, e. g., phaseolin from adzuki beans and from kidney beans, are tested they are found to be immunologically different. These and many other observations with numerous preparations of vegetable and animal proteins, led Wells and Osborne to the conclusion that the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule used as antigen. There is also much evidence to the same effect given by other immunologic reactions.

[8] See also Fink: J. Infect. Dis., 1919, 25, p. 97.

The observations now to be presented have been made with a series of proteins from various beans, prepared in the Protein Investigation Laboratory, Bureau of Chemistry, of the U. S. Department of Agriculture, the chemical properties of which have been studied.[9] The proteins tested have been the alpha and beta globulins of the navy bean (Phaseolus vulgaris), mung bean (P. aureus), adzuki (P. angularis), the lima bean (P. lunatus), and the Georgia velvet bean (Stizolobium deeringianum). From these beans a large proportion of the protein can be extracted with neutral 3% sodium chloride solution, and this protein is readily separated into two fractions, designated as alpha and beta globulins.

The alpha globulins are precipitable from their saline solutions by ammonium sulfate at relatively low concentrations, ranging from 0.2 of saturation in the case of the mung bean to 0.4 in the case of the Georgia velvet bean. The precipitation of the beta globulins ranges from 0.4 of saturation (adzuki bean) to 0.88 (navy bean). Intermediate fractions consisting of a mixture of the alpha and beta globulins are usually obtained. The beta fraction is usually much larger than the alpha fractions.

In general, these fractions seem to be quite distinct from one another, the most striking feature being that the alpha globulins have a much higher sulfur content. Generally, with the exception of the lima bean globulins, the beta globulins have more nitrogen. The differences in composition are inconstant (table 1). In most cases the alpha globulins have much lower coagulation temperatures, and are more easily denatured than the beta globulins. They are also salted out at a lower concentration of ammonium sulfate. A further characteristic difference between these two classes of globulins has been recently shown.[10] It was found that without exception the alpha globulins, which precipitate with ammonium sulfate before the beta globulins, have the higher isoelectric points.

An interesting difference between the alpha and beta globulin of the Georgia velvet bean lies in the behavior of these proteins toward Hopkins and Cole's reagent for the qualitative test for tryptophan. The alpha globulin gives a strong positive test for tryptophan, but the beta globulin gives no trace of the reaction. So far as known no other vegetable globulin has been shown to be entirely lacking in this amino acid

[9] Jones, Finks and Gersdorff: J. Biol. Chem., 1922, 51, p. 103. Jones, Gersdorff, Johns, and Finks: Ibid., 1922, 53, p. 231. Waterman; Johns and Jones: ibid., 1923, 55, p. 93. Johns and Waterman: J. Biol. Chem., 1920, 42, p. 59; ibid., 44, p. 303.

[10] Csonka, Murphy and Jones: J. Am. Chem. Soc., 1926, 48, p. 763.

We will not give in detail the large number of tests made with each protein, thirty or fifty with each, but will save space by summarizing the results of the observations made with the proteins of each species of seeds. In our experiments the anaphylaxis shock reaction was first used for purposes of orientation before applying more exact methods. Here we followed the technic and criteria employed in the anaphylaxis work reported in the studies on vegetable proteins cited previously.[7] In the direct anaphylaxis shock experiments, unless otherwise specified, the sensitizing dose was 5 mg., and the intoxicating dose 50 mg. Unless otherwise stated all injections were made into the peritoneal cavity, and the period of sensitization was usually about 15 to 20 days before reinjection. Use has also been made to some extent of the complement fixation reaction, and in a few experiments the anaphylactic reaction

TABLE 1

Average Chemical Composition of the Alpha and Beta Globulins of Several Beans

	Georgia Velvet Bean Globulins		Navy Bean Globulins		Lima Bean Globulins		Mung Bean Globulins		Adzuki Bean Globulins	
	Alpha	Beta	Alpha	Beta	Alpha	Beta	Alpha	Beta	Alpha	Beta
C	53.08	53.16	53.81	52.56	53.65	52.72	52.98 53.54 53.95 54.05	52.86	52.75	53.57
H	6.81	6.79	6.86	6.81	6.65	6.72	6.91	6.88	6.97	6.79
N	16.66	17.27	15.73	16.13	15.55	14.81	15.74	16.67	15.64	16.46
S	0.91	0.45	1.36	0.33	1.27	0.35	1.48	0.42	1.25	0.40
Arginine	7.19	8.18	6.87	6.36	5.69	5.07	5.13	7.56	5.45	7.00
Histidine	1.24	3.37	0.85	2.36	3.71	2.62	3.31	2.02	2.25	2.51
Lysine	8.32	8.51	10.69	9.42	7.84	8.53	6.08	9.29	8.30	8.41

has been observed by its effect on the contraction of the bronchial musculature by a method which will shortly be published by J. H. Lewis and K. K. Koessler. Especially valuable has been found the uterine strip method of Dale, which was used after the other tests as the most delicate and accurate measure of specificity.

ADZUKI BEAN PROTEINS

By means of the direct anaphylaxis shock test, our preparations of the alpha and beta adzuki globulins cannot be distinguished from one another. Of five guinea-pigs sensitized with beta globulin, four reacted fatally and one moderately when given 50 mg. of alpha globulin intraperitoneally. Of eight guinea-pigs sensitized with alpha globulin, six reacted fatally to 50 mg. of beta globulin, and one moderately; one died from 10 mg. beta globulin. In order to determine more closely the

relation of these two preparations, two guinea-pigs were sensitized each with alpha and beta adzuki globulin, and then desensitized to the heterologous proteins by giving the first dose of 10 mg. subcutaneously, followed by 10 mg. and then 40 mg. intraperitoneally at 24 hour intervals. When given 50 mg. of the homologous protein 48 hours later, they gave but slight reactions, indicating that the heterologous protein had almost completely desensitized the animal, a fact which suggested that these two preparations are antigenically identical, rather than that they represent mixtures of two antigens in differing proportions.

Complement fixation tests again supported the same conclusion, since antiserums for alpha and beta globulin react in almost identically the same dilution, whether tested with the homologous or the heterologous
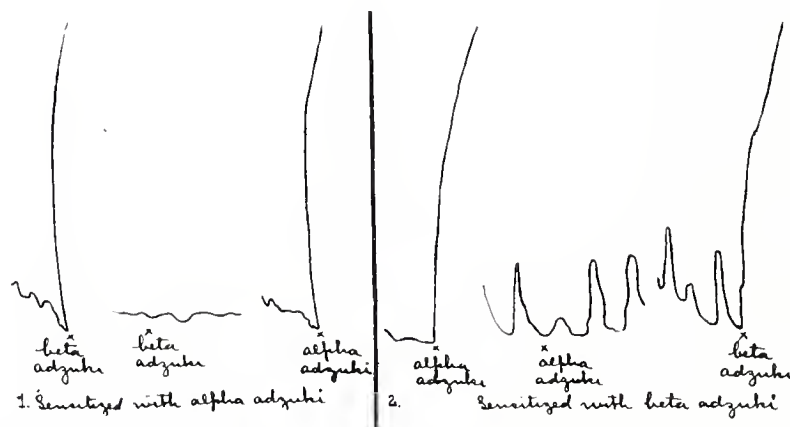
Fig. 1.—Reactions of uterine strip from guinea-pig sensitized with alpha globulin from adzuki bean.
Fig. 2.—Reactions of uterine strip from guinea-pig sensitized with beta globulin from adzuki bean.

preparation. This is also supported by the fact that these two globulin preparations also react about alike with antiserum for either alpha or beta mung globulin.

However, the delicate and specific uterine strip anaphylaxis reaction, established that although these two protein preparations are either mixtures or closely related proteins, nevertheless they can be distinguished. As shown by figure 1, a uterine strip of a guinea-pig sensitized to alpha adzuki reacted strongly with beta adzuki, but nevertheless when completely desensitized to beta adzuki this strip still reacted vigorously to alpha adzuki. A similar result was obtained when the order of reaction was beta-, alpha-, beta-adzuki (fig. 2). This experiment shows conclusively that there are two distinct antigens present in the two prepa-

rations of alpha and beta adzuki under examination, despite our failure to distinguish them by the other methods of testing immunologic specificity.

Comparison with the Globulins of Other Species of Legumes.— These experiments bring out a close relationship between adzuki and mung globulins. Six animals sensitized with beta adzuki globulin were

TABLE 2

Anaphylaxis Tests: Adzuki Bean β Globulin Against Other β Globulins *

	Sensitized with Beta Globulin, 1 to 2 mg.	2nd Injection Beta Globulin, 50 mg.	Reaction	Protection Reaction
1	Adzuki	Adzuki	Died in 20 minutes	
2		Adzuki	Died in 20 minutes	
3		Adzuki	Died in 20 minutes	
4		Mung	Died in 3 hours	
5		Mung	Died in 13 minutes	
6		Mung	Died in 20 minutes	
7		Mung	+++	±
8		Mung	Died in 25 minutes	
9		Mung	Died in 30 minutes	
10		Navy	0	+
11		Navy	0	Died in 30 minutes
12		Lima	±	Died in 95 minutes
13		Lima	0	++
14		Georgia	0	+
15		Georgia	0	Died in 75 minutes
16		Adzuki	Died in 105 minutes	
17	Lima	Adzuki	0	++
18		Adzuki	0	++
19	Mung	Adzuki	Died in 30 minutes	
20		Adzuki	Died in 20 minutes	
21		Adzuki	Died in 23 minutes	
22		Adzuki	Died in 40 minutes	
23		Adzuki	Died in 55 minutes	
24		Adzuki	Died in 50 minutes	
25	Navy	Adzuki	0	+
26		Adzuki	0	±
27	Georgia	Adzuki	0	++
28		Adzuki	0	++
29	Phaseolin	Adzuki	+	Died in 1 hour
30		Adzuki	+	Died in 1 hour

* All injections were made intraperitoneally. The severity of the reactions is indicated by signs with the following significance: 0, no reaction; —, no test made; ±, doubtful reactions, probably not a true anaphylaxis; +, slight reactions, probably true anaphylaxis; ++, moderate typical reactions; +++, severe reactions; ++±, moderate to severe reactions.

given 50 cc. beta mung globulin, and five died in anaphylactic shock, four of them in less than 30 minutes; one reacted very severely and was found later to be desensitized to beta adzuki globulin. In the reversed experiments, six guinea-pigs sensitized to beta mung globulin gave prompt fatal reactions to beta adzuki globulin.

This anaphylactic identity is confirmed by the complete lack of any crossed reactions when the globulin of navy, lima and Georgia velvet

beans are injected into animals sensitized with beta adzuki globulin, or when the experiments are carried out in the reverse order. These differences are shown in table 2.

Control tests with the uterine strip method confirm these results, for it was found that reactions were given to mung proteins after adzuki sensitization, and the reverse. For example (fig. 3), a uterine strip from a guinea-pig sensitized to beta adzuki globulin gave no reaction to alpha or beta Georgia velvet globulin, after which it reacted to alpha mung and to beta mung, after which it was found to be desensitized to alpha adzuki but still somewhat reactive to beta adzuki. In another experiment the uterus sensitized to alpha adzuki globulin gave a positive reaction to alpha mung globulin, after which it was found to be desensitized to beta adzuki, alpha and beta mung globulins. When
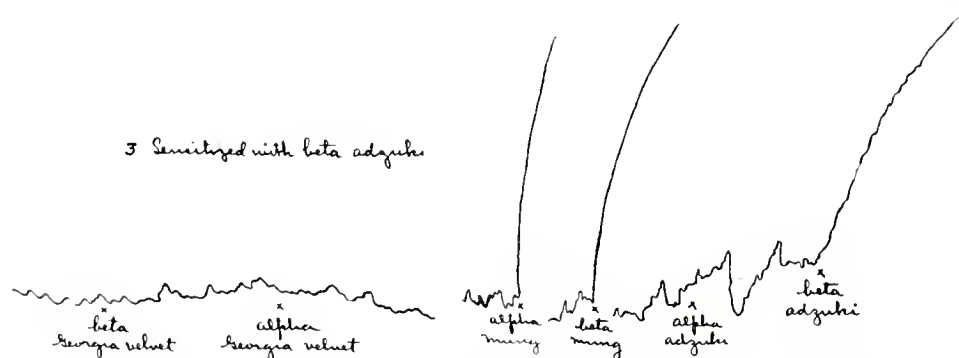
Fig. 3.—Reactions of guinea-pig uterine strip sensitized with beta adzuki globulin.

sensitized with beta mung globulin, a uterine strip gave reactions with both alpha and beta adzuki globulins, after which it was not reactive to alpha mung, although still reactive to beta mung globulin. In other experiments (fig. 4 a and b), uteri sensitized to alpha adzuki gave reactions to alpha mung after which they were found to be desensitized to alpha adzuki. Similarly beta mung globulin reacted with and desensitized uteri sensitized with beta adzuki. When the reactions were reversed, uteri sensitized to alpha and beta mung gave reactions with alpha and beta adzuki globulins respectively, and were thereby desensitized to the homologous proteins.

On the other hand, the uterine strips sensitized to either adzuki or mung globulins do not react when tested with the globulins of lima, navy or Georgia velvet bean.

Complement fixation tests give corresponding results, cross reactions being obtained with alpha and beta mung and alpha and beta adzuki

globulins and the corresponding antiserums, and usually in about the same dilutions when homologous and heterologous antigens and antiserums are tested, although some irregular quantitative variations were noted. Also there is an absence of complement fixation with navy, lima, and alpha globulin of Georgia velvet bean.

To summarize, there seems to be antigenic similarity but not identity of the alpha and beta globulin preparations of adzuki bean tested by us. These two globulins seem either identical with or closely related to the alpha and beta globulins of the mung bean. On the other hand, the alpha and beta globulins of adzuki bean seem to be entirely distinct from the globulins of navy, lima and Georgia velvet bean.
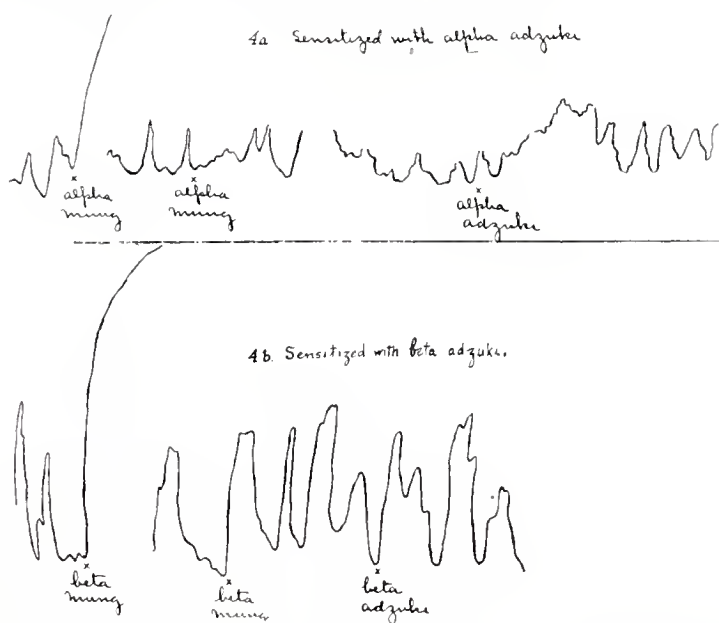
Fig. 4.—Reactions of guinea-pig uterine strips sensitized: a, with alpha adzuki globulin; b, with beta adzuki globulin. Desensitization followed reactions with mung globulins.

MUNG BEAN GLOBULINS

These seem to be indistinguishable from the adzuki bean globulins, not only in the interreactions obtained with these proteins with immune serums and in anaphylaxis tests, but also in respect to other proteins. That is, animals sensitized to mung globulins do not react with the proteins of navy, lima or Georgia velvet bean globulins, and these heterologous proteins do not desensitize the animals to the mung bean globulins. The results were quite the same whether the anaphylactic shock method or the uterine strip method was used, and it is not necessary to repeat in detail the several experiments. In addition, in

one sensitized guinea-pig the bronchospastic test of anaphylaxis of Koessler and Lewis [11] was applied. As shown by figure 5, a guinea-pig sensitized with alpha globulin of mung bean gave no reactions to wheat proteins or to alpha or beta globulins of the Georgia velvet bean, but after these tests was still highly sensitive to the beta globulin of mung beans.

As with the alpha and beta globulins of adzuki beans, the corresponding alpha and beta globulins of mung bean could not be distinguished from one another by simple anaphylaxis shock tests. Animals sensitized with either protein gave promptly fatal reactions with the other. Complement fixation tests confirmed these anaphylaxis tests.

Fig. 5.—Bronchiospastic reaction in guinea-pig sensitized with alpha globulin of mung bean, and treated with wheat proteins, Georgia velvet bean globulins and then beta mung globulin.

Antiserum for mung protein gave strong reactions with adzuki globulins, practically the same whether alpha or beta globulins were used in either the immunization or in the complement fixation tests. The only exception was furnished by alpha mung globulin, which reacted but slightly with the antiserum for beta mung and alpha adzuki globulin, and not at all with antiserum for beta adzuki in one set of experiments, but with the antiserums obtained with another set of animals no such distinction could be made, the results agreeing closely with those obtained by the anaphylaxis tests.

However, the crucial saturation test with the sensitized uterine strip showed that the alpha and beta mung globulins can be distinguished from

[11] In press, Arch. Int. Med.

each other by this method. That is, a uterine strip sensitized to alpha mung reacts to beta mung, but after being desensitized to beta mung it still reacts with alpha mung. Corroborative results are given when the experiments are carried out in the reverse order, i. e., beta-alpha-beta mung globulin (fig. 6 a and b).

The close relationship between the adzuki and mung proteins found by immunologic tests is interesting in view of the close agreement between the figures for the elementary composition of these proteins and their nitrogen distribution as determined by the Van Slyke analysis.
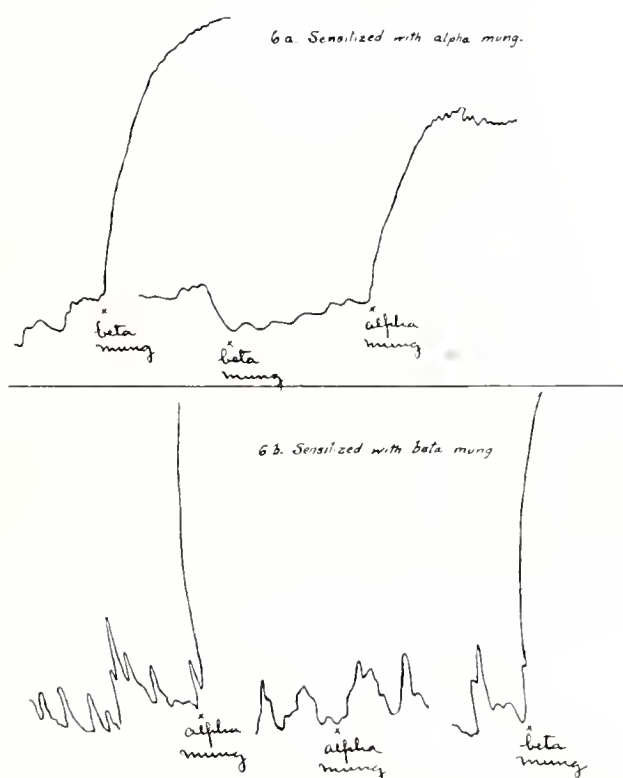
Fig. 6.—Reactions of guinea-pig uterine strips sensitized: a, with alpha mung globulin; b, with beta mung globulin. Alpha and beta globulins distinguished by saturation tests.

Of further interest is the fact that Dr. C. V. Piper, agrostologist in the Bureau of Plant Industry, who has made a special botanical study of these two beans, has found them to be so closely related that he has proposed that they be classified, not as phaseolus, as they are at present, but under a separate genus or subgenus.

To summarize, alpha and beta globulin preparations from mung bean are closely related according to immunologic tests, but the uterine strip reaction shows them to contain distinct antigens. The similarity or

identity of these two proteins and the corresponding globulins from adzuki beans is confirmed. They are immunologically distinct from the globulins of lima, navy and Georgia velvet beans.

GEORGIA VELVET BEAN GLOBULINS

These stand out among the globulins tested in that they seem to be entirely distinct from all the others. No reactions whatever were obtained with velvet bean globulins in animals sensitized with lima, navy, adzuki or mung bean proteins, or with the reactions performed in the opposite direction, and the animals were not protected by the hetero-logous proteins from reacting with homologous proteins. The same results were obtained with the uterine strip method.

Comparison of the alpha and beta globulin preparations from the Georgia velvet bean indicated that these were not antigenic equivalents. Although guinea-pigs sensitized with alpha globulin gave severe or

TABLE 3
Georgia Bean Globulin Saturation Experiment

Sensitized with Georgia Bean Globulin	Second Injection	Third Injection	Result
Alpha	Beta, 5 + 50 mg.	Alpha, 50 mg.	Moderate
Alpha	Beta, 10 + 50 mg.	Alpha, 50 mg.	Moderate
Alpha	Beta, 10 + 50 mg.	Alpha, 50 mg.	Died, 30 minutes
Beta	Alpha, 5 + 50 mg.	Beta, 50 mg.	Slight
Beta	Alpha, 10 + 50 mg.	Beta, 50 mg.	Moderate
Beta	Alpha, 10 + 50 mg.	Beta, 50 mg.	Slight

fatal reactions with beta globulin, those sensitized with beta did not give fatal reactions with alpha, and on recovery they were found not fully desensitized to beta globulin. Therefore an experiment was carried out in which the animals were thoroughly saturated by giving first 5 or 10 mg. of the heterologous protein, followed in a few hours by a 50 mg. dose of the same protein. The results are given in table 3.

All gave slight reactions to the first dose of 5 to 10 mg., of hetero-logous protein, but little or no effect with second 50 mg. dose of the heterologous protein on the same day. Forty-eight hours later some reactions were obtained with the homologous protein, but severe reactions only once, therefore, there seems to be some protection but not complete.

These experiments suggest that there are two different proteins, admixed in each of our preparations of Georgia bean globulins. Uterine strip experiments corroborate this conclusion, for reactions were obtained with the heterologous proteins, but by such reactions the strip was not fully desensitized to the homologous proteins (fig. 7).

navy bean globulins

These two proteins, called conphaseolin and phaseolin, which are equivalent respectively to the alpha and beta globulins, were found to be distinct from the proteins of mung, adzuki, and Georgia velvet beans by all three methods of testing, but they are not fully distinct from the
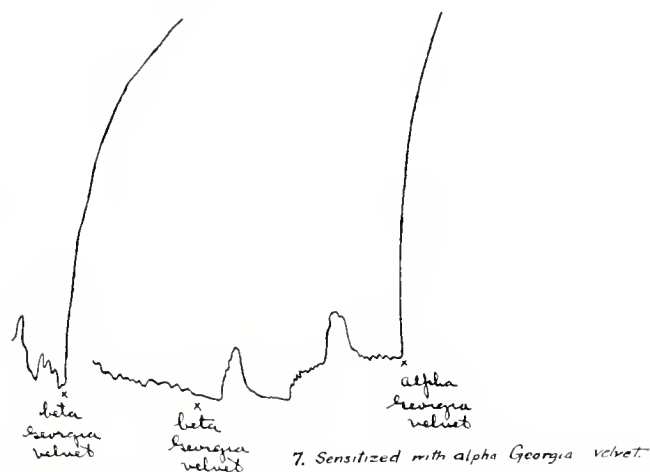
Fig. 7.—Reactions of guinea-pig uterine strip sensitized with alpha globulin from Georgia velvet bean, showing failure of complete desensitization by heterologous fraction.
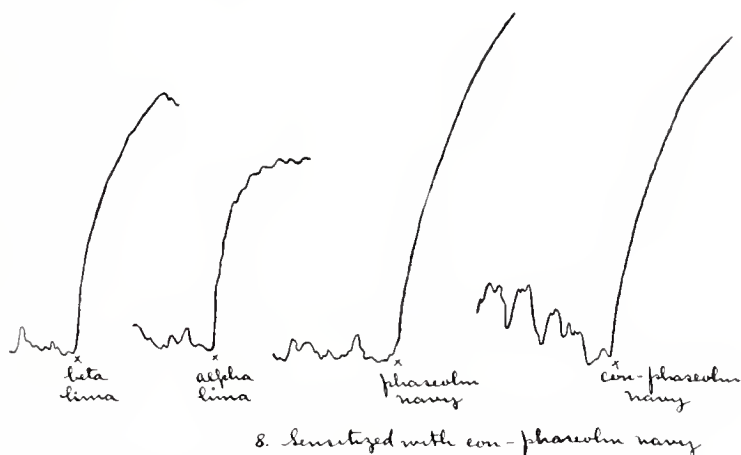
Fig. 8.—Reactions of guinea-pig uterine strip sensitized with conphaseolin (analogous to alpha globulin) of navy bean.

proteins of the lima bean. Invariably guinea-pigs sensitized to beta globulin of lima bean would react with phaseolin, to a greater or less degree, but never severely, and the reaction produced only part desensitization or none at all. Our results with this protein in anaphylaxis tests were unsatisfactory, as the phaseolin forms a mucilaginous solution in

0.1% NaOH, apparently but little being in true solution. However, as animals sensitized with phaseolin gave constantly severe to moderate anaphylactic reaction with the beta globulin of lima bean, this inter-reaction seems to be definite, although not complete since heterologous reactions did not desensitize the animals to the homologous proteins. A second preparation of phaseolin, which was somewhat more soluble than the one first used, also gave reactions in guinea-pigs sensitized with beta lima globulin, fatal in two of six experiments, although the surviving animals were still strongly sensitive to the lima globulin.

Likewise, tests by the uterine strip method showed a similar relationship. For example, the uterus of a guinea-pig sensitized to conphaseolin reacted with the beta globulin of the lima bean, then with alpha lima globulin, then with phaseolin, after which it was still reactive to con-phaseolin (fig. 8). A uterine strip sensitized with beta navy globulin (phaseolin) reacted to alpha navy, but after desensitization was still reactive to beta navy globulin. A strip sensitized to beta navy reacted to beta lima but was still reactive to beta navy globulin (fig. 9). These tests show that despite the close relationship exhibited between lima and navy bean globulins by immunologic tests, nevertheless they are definitely distinguishable from each other by delicate methods.

Complement fixation tests gave quite the same results. That is, antiserums for lima bean globulins gave reactions with navy bean globulins, but not so strongly as with the lima bean globulins.

Immunologic comparison of the conphaseolin and the phaseolin gave about the same results as were obtained with the alpha and beta globulins of the velvet bean. That is to say, sensitization with either made the animal reactive to the other, but such heterologous reactions did not completely desensitize to the homologous protein. The results were the same in shock experiments and in uterine strip tests (fig. 10).

To summarize, the alpha and beta globulins of navy bean (con-phaseolin and phaseolin) are readily shown to be distinct antigens. They are immunologically distinct from the globulins of mung, adzuki and Georgia velvet bean, but not from the globulins of the lima bean. Here the relationship is close, but the most delicate tests show that the lima and navy bean globulins are distinguishable from each other.

LIMA BEAN GLOBULINS

These were immunologically distinct from the globulins of adzuki, mung and Georgia velvet bean, but, as stated above, showed some relation to the globulins of the navy bean. When the alpha and beta glob-

ulins of lima beans were compared with each other, it was found that our preparations were not entirely distinct from each other, but nevertheless were immunologically distinguishable. The alpha globulin preparation, however, was not sufficiently soluble in the body fluids of the guinea-pig to give satisfactory results in shock experiments. Uterine strip tests gave the following results:
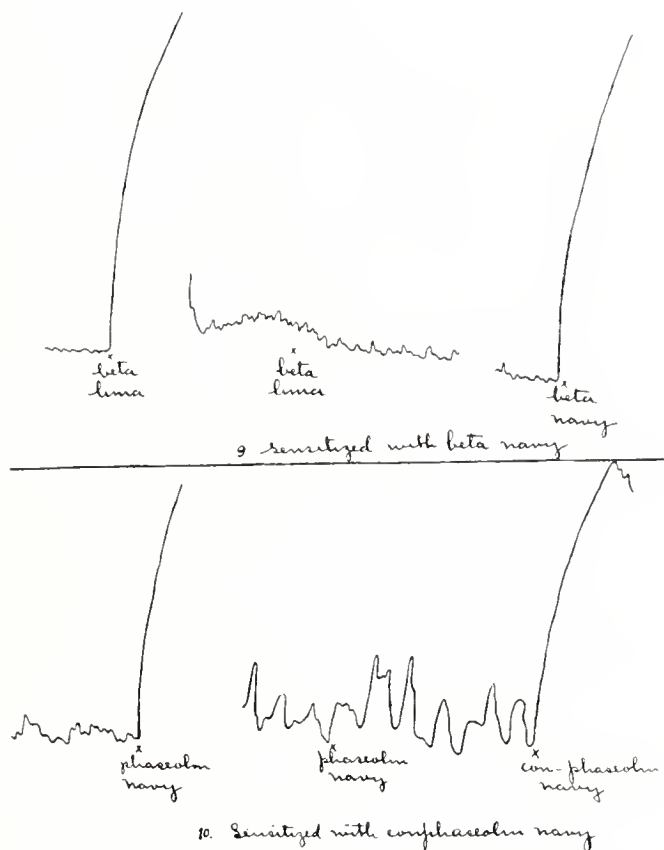
Fig. 9.—Reactions of guinea-pig uterine strip sensitized with phaseolin (beta globulin) of navy bean, distinguishing beta globulins from navy and lima beans.

Fig. 10.—Reactions of guinea-pig uterine strip sensitized with conphaseolin of navy bean, showing distinction from phaseolin.

Alpha lima sensitization.—The uterus reacts to both alpha and beta navy bean globulin, and still reacts to beta lima globulin, after which it is not reactive to alpha lima globulin. Conversely, the uterus of a guinea-pig sensitized to alpha navy bean globulin reacts with both alpha and beta globulins of lima bean, and is still reactive with alpha navy bean globulin (figs. 11 a, b).

Beta lima sensitization.—The uterus did not react to alpha or beta adzuki globulin, it then reacted with alpha navy globulin, failed to react in turn to beta navy, and alpha lima, but was still reactive to beta lima globulin (fig. 12). In another experiment the results were as follows: Alpha and beta adzuki globulin, no reaction; alpha navy globulin, reaction; beta navy, reaction; alpha lima, reaction.

These results indicate the presence in our alpha and beta lima globulin preparations of common and distinct antigens; i. e., each preparation probably consists of a mixture of two proteins. This conclusion is
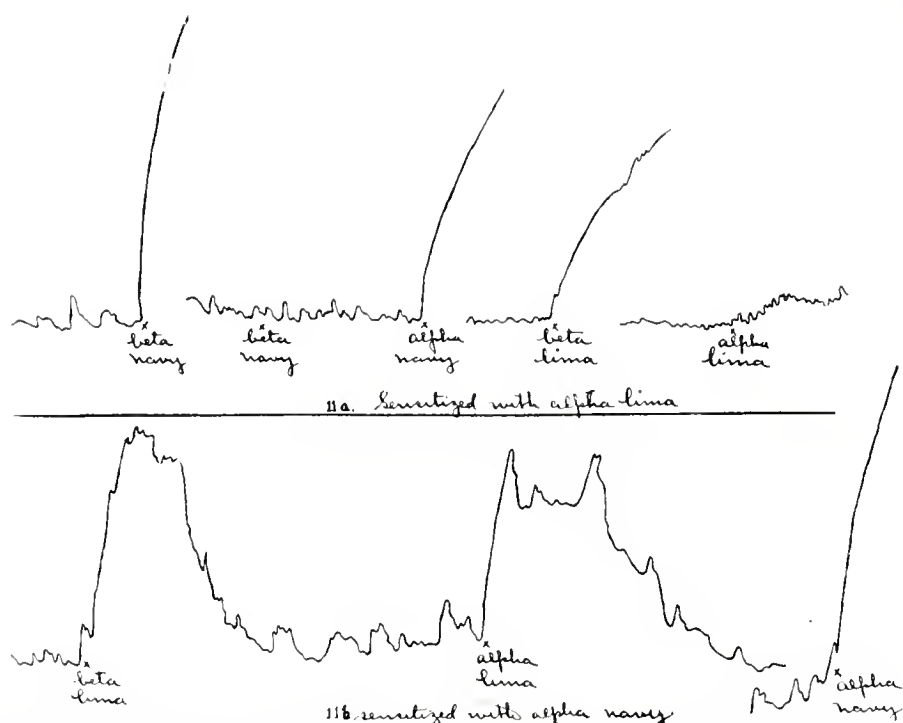
Fig. 11.—Reactions of guinea-pig uterine strips sensitized: a, with alpha globulin from lima bean; b, with alpha globulin from navy bean.

supported by our complement fixation tests, which show that the antiserum for beta lima globulin reacts much less strongly with alpha than with beta lima globulin, although the antiserum for alpha lima globulin gives about equal reactions with alpha and beta lima globulin.

SUMMARY

Immunologic studies have been made of the alpha and beta globulin fractions of several leguminous seeds, namely, navy, mung, adzuki, lima and Georgia velvet beans. These two protein fractions are chemically

distinct from each other as obtained from each of the five species of beans, the most marked difference being that the alpha globulins have a higher sulfur and usually a lower nitrogen content. The alpha and beta fraction of the five different beans when compared with one another show some chemical similarities and some differences.

Immunologic tests brought out more clearly these relationships and differences. Anaphylaxis shock reactions, uterine strip anaphylaxis reactions and complement fixation reactions were used with all these proteins, and in general confirmed each other, but the uterine strip reaction was found to give the most accurate differentiation of the proteins, especially when these were admixt with one another. Only by this method was it possible to show conclusively that our several
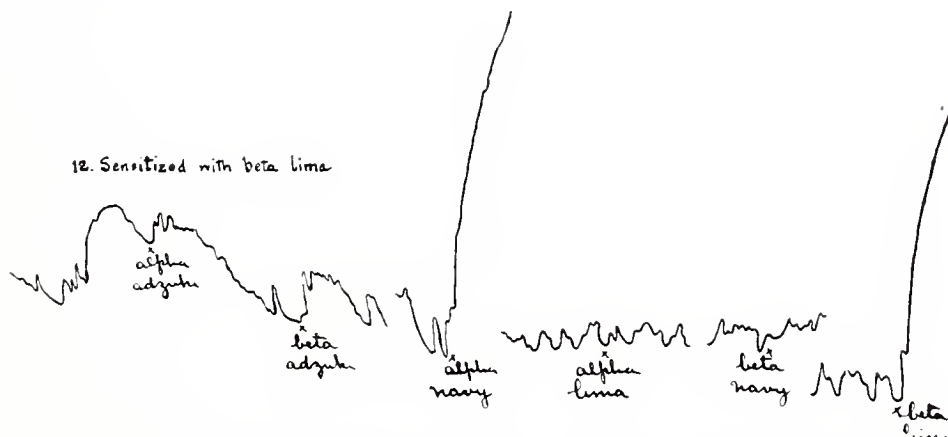
Fig. 12.—Reactions of guinea-pig uterine strip sensitized with beta globulin from lima bean, indicating presence in alpha and beta preparations of common and distinct antigens.

preparations of alpha and beta globulins contain admixtures of two distinct antigens, confirming the chemical evidence that there are two distinct globulins in each species of bean.

The globulins from adzuki and mung beans were found to be distinct from the globulins of navy, lima and Georgia velvet beans, but the alpha and beta globulins of adzuki and mung beans seem to be either identical with or similar to each other. This immunologic similarity is found to correspond to a close chemical similarity according to such methods as are available for the study of protein chemistry.

Georgia velvet bean globulins were found to be distinct immunologically from the globulins of the four other species of beans tested. This may be related to the fact that chemically these globulins have a higher nitrogen content than the others, and a larger proportion of alpha

globulin. Botanically the Georgia velvet bean is one of the Stizolobia, the other four beans belonging to the Phaseolus group.

The globulins of the lima and navy beans, although closely related to each other, were found to be distinct from the globulins of mung, adzuki and Georgia velvet beans. Nevertheless the lima and navy bean globulins are distinguishable from each other by the delicate uterine strip method.

BACTERIAL FILTERS

S. P. KRAMER

From the U. S. Hygienic Laboratory, Washington, D. C.

We have come to divide bacteria and viruses into filtrable and non-filtrable, and we have come to think that those organisms, visible or invisible, which are smaller than the pores of our filter are filtrable. That size, however, cannot be the sole criterion we have known from the behavior of certain aniline dyes.[1] Thus Victoria blue, a basic dye, will not pass a Berkefeld filter while Congo red, an acid dye, will readily pass through the same filter. Now it happens that the filters which we use in bacteriologic practice, namely, sand, porcelain, and diatomaceous earth, are all of them some form or compound of silicic acid, so that really when one speaks of a filtrable organism, dye or other colloid, one ought to say filtrable through siliceous filters.

One may speak of a filter when it is in action as a suspension of the material of which the filter is composed, in the fluid which is being filtered. Now silica has a definite negative charge and it may be that if one constructs a filter of a material of charge opposite to that of silica, one might find that bacteria or colloids which are filtrable through silica filters are nonfiltrable through such other filters, and vice versa.

Accordingly, filters were made of plaster of Paris and experiments with various dyes and viruses were made. Thus Victoria blue which does not pass a Berkefeld or siliceous filter readily passes through a filter made of plaster of Paris; while Congo red which readily passes through the Berkefeld filter does not pass through a plaster of Paris filter. If, however, we make a dilute solution of Congo red and render it very slightly acid, thereby changing the color to blue, we find that the blue dye does not pass trough the Berkefeld filter but does pass through the plaster of Paris filter. In other words, by reversing the electrical charge one has reversed the filtrability.

[1] In 1916 I showed before the Research Society of Cincinnati that any filter made of siliceous material, sand, porcelain, powdered glass, colloidal silica, or diatomaceous earth would absorb basic dyes, while acid dyes would pass through.

Stuart Mudd (Am. J. Physiol., 1922, 63, p. 429) described the surface of the pores of a Berkefeld filter as the site of an electrical potential difference, a Helmholtz double layer, in which the wall of the filter carried a negative charge and the liquid a positive charge; and predicted and found that when suspensions were filtered through such a filter, positively charged particles were absorbed and retained by the filter.

A preliminary report on the subject of bacteria filters was published by me in Gen. Physiol., 1926, 9, p. 811.

Acid and basic dyes should be used to test filters. In this work, any Berkefeld filter which does not completely remove Victoria blue from the solution is rejected. The gypsum filter is tested with Congo red.

Now when we came to consider the nature of plaster of Paris a very interesting phenomenon was found. Plaster of Paris is supposed to be calcium sulfate but when filters were made of calcined chemically pure calcium sulfate, it was found that such filters had no action on any of the colloid dyes used. Both Victoria blue and Congo red readily passed through such filters. Calcium sulfate is neutral and without charge. Then it was found that the plaster of Paris of commerce contained up to 5% of calcium carbonate, and when calcium carbonate was added to our chemically pure calcium sulfate and filters made from this mixture, such filters acted as did the filters made of commercial plaster of Paris. The calcium carbonate is alkaline and has a positive electrical charge. It is probable that the calcium sulfate in our filters acts as a binder for the calcium carbonate and that it is the calcium carbonate which is the active absorbing component of our filter.

It was thought that if a filter was made of plaster of Paris and silica or diatomaceous earth intimately mixed before setting, that we might produce a filter which would remove both positively and negatively charged dyes and colloids. When this was done, however, it was found that such filters were neutral and that neither category was filtered out. Evidently the calcium carbonate and silica neutralized each other. If, however, the filter was made so that the core was a Berkefeld filter with a cortex of plaster of Paris poured over this, so that the contact of materials was only at the joint surface, then it was found that dyes, viruses and colloids of both positive and negative charge could be removed by filtration through this apparatus. The outer layer when made of plaster of Paris removed the acid or negative particles, and the inner or siliceous core removed the basic or positive particles.

It was found that one could increase the efficiency of the basic or gypsum filter by incorporating in the plaster of Paris up to 25% of magnesium oxide, calcined at 1300 C.

Experiments have been made with socalled filtrable microorganisms and viruses that were available and a concise report of these experiments up to date will here be given:

The bacteriophage of Staphylococcus aureus which passes through the Berkefeld filter does not pass the filter of plaster of Paris. Vibrio percolans, as described by Stuart Mudd, passes through the Berkefeld filter but does not pass the gypsum filter. The same is true of vaccine virus and the virus of rabies.

The following tests were made by Mr. H. H. Allard of the United States Department of Agriculture with the sap of mosaic disease of tobacco filtered through several types of gypsum filters. These tests were made May 19, 1926:

Test 1.—10 plants inoculated with the unfiltered mosaic sap: 9 plants became mosaic-diseased.

Test 2.—10 plants inoculated with Berkefeld plus plaster of Paris filter: none became mosaic-diseased.

Test 3.—10 plants inoculated with virus passing through plaster of Paris filter alone: none became diseased.

Test 4.—10 plants inoculated with mosaic sap filtered through a filter made of plaster of Paris plus 2% calcium carbonate ($CaCO_3$): none became diseased.

On May 29 these same filtrates were again used with the following results:

Test 5.—10 plants inoculated with the unfiltered mosaic sap gave 7 plants mosaic disease.

Test 6.—10 plants inoculated with the sap filtered through a Berkefeld plus plaster of Paris filter: none became diseased.

Test 7.—10 plants inoculated with plaster of Paris filtrate: none became diseased.

Test 8.—10 plants inoculated with sap filtered through a filter made of plaster of Paris plus 2% calcium carbonate ($CaCO_3$): none became diseased.

It was also found that one could remove various bacterial toxins by filtration. Diphtheria toxin—and by this I mean standard toxin of the U. S. Hygienic Laboratory—when filtered through a filter made of a mixture of plaster of Paris with 25% magnesium oxide calcined at 1300 C., had its general toxic principle removed. The following series of experiments illustrates this:

Guinea-pig 1 (Control).—Weight 400 gm., Nov. 30, 1926, 2 p. m., received diphtheria toxin 10 MLD subcutaneously. Dec. 1, 1926, 6 p. m. Necropsy showed diphtheria.

Guinea-pig 2.—Weight 460 gm., Nov. 30, 1926, 2 p. m., received subcutaneously 1 cc. diphtheria toxin containing 2 L + doses or 100 + MLD which had been filtered through a filter made of plaster of Paris + 25% MgO.

Guinea-pig 3.—Weight 420 gm., received 2 cc. = 200 + MLD diphtheria toxin which had been filtered through a filter made of plaster of Paris + 25% MgO.

Guinea-pig 4.—Weight 550 gm., received 4 cc. = 400 MLD filtered toxin.

Guinea-pig 5.—Weight 540 gm., received 5 cc. = 500 MLD filtered toxin.

All of the guinea-pigs receiving toxin filtered through the plaster of Paris-magnesia filters were alive and well December 16, 1926,[*] except that each guinea-pig had developed an ulcer with denudation of hair at the site of injection. The size of the necrotic area varied directly with the amount of filtered toxin injected.

It was evident that the filter had removed that fraction of the toxin which caused the acute death and neurotic symptoms, leaving the fraction which caused the local tissue changes.

This tissue toxin was removed by filtration through the Berkefeld filter charged with silicic acid gel. Animals which received toxin which

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[*] Observations made after paper was submitted for publication.

had been filtered through both filters in amounts up too 10 L + doses developed no symptom whatever—either local or general.

When diphtheria toxin is filtered through siliceous filters alone the animal dies promptly of general intoxication with doses approximating the lethal dose of unfiltered toxin.

Botulinus toxin is also rendered inoccuous by filtration through filters made of plaster of Paris and magnesia.

Abrin, the toxin from Jequerity seed, is removed by filtration through the same basic filter and is unaffected by filtration through siliceous filters.

Tuberculin when passed through a Berkefeld filter or a Berkefeld filter charged with colloidal silica is still lethal to tuberculous guinea-pigs.

Tuberculin passed through a filter made of plaster of Paris with 25% magnesium oxide is no longer lethal to tuberculous pigs. This is illustrated by the following experiments:

Guinea-pig A.—Weight 450 gm., Oct. 12, 1926, received 2 mg. tubercle bacilli culture H. 37 in the axilla. Nov. 4, 1926, this guinea-pig received at 10:30 a. m. 5 cc. of a solution of tuberculin, equivalent of 0.625 cc. of old Koch tuberculin, and died the following night. Necropsy showed intense tuberculin reaction.

Guinea-pig B.—Weight 430 gm., Oct. 12, 1926, received a like amount of tubercle bacilli. Nov. 31, 1926, 10 a. m., guinea-pig B received 5 cc. of the above solution of tuberculin which had passed through a Berkefeld filter charged with colloidal SiO_2. This guinea-pig died at 2:30 p. m. of the same day with a marked tuberculin reaction.

Guinea-pig C.—Weight 420 gm., Oct. 12, 1926, received a like amount of tubercle bacilli. Nov. 30, 1926, 10 a. m., guinea-pig received 5 cc. of the same tuberculin solution which had passed through a filter of plaster of Paris with 25% MgO. The guinea-pig was unaffected and remained as before injection. Dec. 2, 1926, 9:10 a. m., guinea-pig C received 5 cc. of the same tuberculin filtered through the Berkefeld filter charged with colloidal silica and died nine hours later with a marked tuberculin reaction.

Tetanus toxin on the other hand is unaffected by filtration through any gypsum filter, but as has long been known is removed in limited quantities by siliceous filters. If one takes the Berkefeld filter and first draws through it freshly gelled pure colloidal silica, one obtains a siliceous filter through which one may pass tetanus toxin and obtain a filtrate of which one may inject up to an equivalent of 50 MLDs for the guinea-pig. With the ordinary Berkefeld filter I have never been able to succeed in producing a filtrate of which one might inject more than 10 MLDs.

Experiments were also made of filtering with both classes of filters watery infusions of the various neurotoxic drugs with the idea of

removing, if possible, the neurotoxic components. A large number of such experiments were done but they will be recorded in another communication. Here, however, I wish to state that so far as I have gone, both with the bacterial toxins and the toxic components of the various neurotoxic drugs, the following rule has held: namely, that all narcotizing toxins and drugs are filtered out with the basic or positively charged filter and the spasm-producing toxins and drugs come out with the siliceous or acid or negatively charged filter. And this makes it strongly probable that the respective nerve cells involved in the production of the above two classes of symptoms have a similar though opposite difference in reaction or charge.

SUMMARY

Since all bacterial filters hitherto used have been made of siliceous material of negative electrical charge, it was thought that one might by using a material of opposite charge, obtain a filter which would remove bacteria, viruses and colloids which have hitherto been classified as filtrable. By the use of calcium carbonate and magnesium oxide of positive electrical charge, this has been accomplished.

OCCURRENCE OF BACTERIUM COLI OF INTESTINAL ORIGIN ON HANDS OF FOOD HANDLERS

W. A. Buice, H. C. Sehested and R. B. Dienst

From the Department of Zoology and Hygiene, Baylor University, Waco, Texas

The purpose of the present paper is to show something of the frequency of the occurrence of Bacterium coli of intestinal origin on the hands of food handlers in restaurants, cafes, lunch counters, sandwich shops and soda fountains. The problem was suggested by seeing two waiters at a lunch counter in a hotel pass back through the lobby of the hotel to the men's room, visit the commodes, and return to the lunch counter without washing their hands. The first duty performed by one of the waiters after returning to the lunch counter was to hand a customer an unwrapped sandwich.

This investigation was made in the public eating places of Waco, Texas, which has a population of about 45,000. On casual inspection the general sanitary conditions of public eating places here appear as good as those in the average American town of this size. Such places are inspected and scored monthly, or oftener, by the city health department. All the tests on the hands of the food handlers were made between 8:00 and 10:00 o'clock in the morning.

Materials and Methods.—Dehydrated culture medium preparations from the Digestive Ferments Company were used: lactose broth, 13 grams per liter of distilled water; Levine's eosin methylene blue agar; and nutrient agar, 1%, for the agar slants. Koser's[1] sodium citrate medium was used for distinguishing the Bacterium coli of intestinal origin from that of vegetable origin.[2] All mediums were sterilized for twenty minutes at fifteen pounds pressure, the tin plates were sterilized by dry heat at 170 C. for two hours, and all incubations were at 37.5 C.

A modification of the Bacterium coli test method in bacteriologic water analysis was used as described in Standard Methods of Water Analysis, published by the American Public Health Association.[3] The

[1] J. Bact., 1923, 8, p. 493; 1924, 9, p. 59; J. Infect. Dis., 1918, 23, p. 377; 1924, 35, pp. 14, 315; J. Am. Water Works Assoc., 1924, 12, p. 200.

[2] Am. J. Pub. Health, 1925, 15, p. 614; 1923, 13, p. 822; Standard Methods of Water Analysis, A. P. H. A., 1923, p. 106; J. Am. Water Works Assoc., 1924, 11, p. 416.

[3] Standard Methods of Water Analysis, A. P. H. A., 1923, p. 92; J. Am. Water Works Assoc., 1925, 14, p. 535.

test of the hands of the individual food andler was made in his place
of employment, while on duty. The procedure was as follows:

Into a large test tube (1 by 8 inches) a small test tube, three inches long,
was placed, inverted, to serve as a fermentation tube. The lactose broth poured
into the large tube was measured so that after sterilization in the autoclave the
small inner tube was filled and there was half an inch of broth in the large
tube. The large tubes were plugged with cotton and sterilized in the autoclave.

Lactose broth was also made up in a 500 cc. filter flask with a side neck. A
rubber nipple was applied to the side neck as a stopper. The flask and contents
were then sterilized in the autoclave.

Tin plates, eight inches in diameter were inverted and sterilized by dry heat.

The fermentation tubes; the flask of broth, and the sterilized tin plates were
then taken to the food handler's place of employment. From the side neck of
the filter flask about 50 cc. of the lactose were poured on the hands of the food
handler while he rubbed them together over the sterilized plate. Thus, the
broth in which he had washed his hands was collected in the tin plate and
immediately poured into a fermentation tube. The contents were well mixed by
rolling the tube between the hands, the tube was numbered and the necessary
information recorded, and the tube returned to the laboratory and incubated.
Each food handler was tested individually in this manner.

After 48 hours of incubation at 37.5 C. the percentage of gas present in the
small inner tube was recorded. If gas were present, broth from this large
tube was streaked on an eosin methylene blue agar plate, which was incubated
24 hours at 37.5 C. and examined for typical Bacterium coli colonies. These
colonies were 2 to 3 mm. in diameter; slightly raised and rarely convex; with
dark almost black centers, three-fourths the size of the colony, by transmitted
light; and dark, often concentrically arranged with greenish metallic sheen
when seen by reflected light.

If such colonies appeared, two isolated ones were marked and portions of
each were transferred to a fermentation tube containing lactose broth, and to a
nutrient agar slant. After incubation for 24 hours, the lactose broth tubes were
examined for gas formation. If gas were formed in the lactose broth tube, and
an abundant growth were present on the corresponding nutrient agar slant,
the test was recorded as positive for the Bacterium coli group.

Those organisms which fermented lactose broth and produced colonies typical
of Bacterium coli on the eosin methylene blue agar were transferred from the
agar slant to Koser's sodium citrate medium and incubated for 4 days at 37.5 C.
Great care was taken that only the organisms were transferred and no medium.
Six inch test tubes one-third full of Koser's sodium citrate medium furnish a
clear fluid medium in which turbidity developed by the growth of organisms
is easily detected.

At the end of four daps, the tubes were examined for growth as indicated
by turbidity. Those organisms which failed to grow in the sodium citrate
medium were regarded as Bacterium coli of intestinal origin. Those which
did develop in the citrate medium were regarded as of nonfecal or vegetable
origin.

The differentiation is based on the fact that colon organisms of
intestinal origin cannot utilize sodium citrate as its only source of carbon
for their metabolic activities, while Bact. aerogenes, which is of vegetable

origin, can use this salt as its source of carbon.[4]   Hence, in Koser's medium it appears that the colon bacterium of fecal origin does not grow, while those of nonfecal origin do grow and produce a turbidity in the otherwise clear medium.

Results.—A total of 337 tests (men, 229 and women, 98) were made on 251 food handlers. Lactose fermenting aerobes were present in 228 tests, 67.65%. Of these, 129, or 38.28% when transferred to eosin methylene blue agar plates produced typical Bacterium coli colonies. After culturing on Koser's sodium citrate medium, four days incubation, only 27, 8.38%, of the total number of tests showed the presence of Bacterium coli of intestinal origin. Of those food handlers examined twice, only one gave a positive test for Bacterium coli of intestinal origin in each examination. Fourteen races or nationalities were represented as shown in table 1.

TABLE 1

Positive Tests for Bacterium Coli Arranged According to Race and Sex

Nationality	Sex	Number Tested	Positive Number	Positive %
American	Male	68	4	5.8
	Female	63	5	7.9
	Total	131	9	6.8
Negro	Male	62	5	8.0
	Female	26	3	11.6
	Total	88	8	9.0
Greek	Male	47	5	10.6
	Female	1	0	0
	Total	48	5	10.4
Mexican	Male	27	1	3.7
	Female	2	1	50.0
	Total	29	2	6.9
German	Male	10	0	0
	Female	5	1	20.0
	Total	15	1	6.6
French	Male and female	2	0	0
Chinese	Male	8	1	12.5
Bohemian	Male	4	0	0
Irish	Male	3	0	0
Syrian	Male	3	0	0
Rumanian	Male	2	0	0
Italian	Male	2	0	0
Swedish	Male	1	1	100.0
Dutch	Male	1	0	0
Total	Male	239	17	7.2
	Female	98	10	10.2
	Total	337	27	8.38

The figures also show that women gave a larger percentage of positive tests for intestinal Bacterium coli than men among the American and negro food handlers. It also shows that the Mexicans had the smallest, and the Greeks the largest percentage of positive tests.

[4] J. Am. Water Works Assoc., 1924, 12, p. 200.

TABLE 2

POSITIVE TESTS FOR BACTERIUM COLI IN RELATION TO AGE OF PERSON

Age in Years	Number of Tests	Positive Tests	
		Number	%
15 to 20...	27	3	11
20 to 25...	76	6	7
25 to 30...	65	5	7
30 to 35...	57	4	7
35 to 40...	56	4	7
40 to 62...	56	5	8

Table 2 shows that there are more positive intestinal Bacterium coli found on food handlers between the age of fifteen and twenty than at other ages. It also shows that at the ages between 40 and 62 the percentage of positive tests again increases.

CONCLUSION

Bacterium coli of intestinal origin was present on the hands of food handlers while at work, in 8.38% of the 337 tests made.

FURTHER STUDIES ON THE ISOLATION AND CULTIVATION OF BACTERIUM ABORTUS (BANG)

I. Forest Huddleson, D. E. Hasley and J. P. Torrey

From the Bacteriological Section, Michigan Agric. Exper. Sta., East Lansing

The study of the physiology of the growth and reproduction of Bacterium abortus which makes its isolation on artificial culture medium possible, is unquestionably one of the most interesting phases of the problem that has been presented to investigators in their study of Bang's abortion disease. The growth and growth requirements of this particular microorganism are unique in that there is no other pathogenic parasite yet studied which is such a peculiar anaerobe; not in the strict sense of the definition of the word, but because it does not show visible growth in a medium exposed to the air on primary isolation, and later, after successive transfers it becomes a true aerobe.

The explanation of the primary growth of the organism on artificial culture mediums has been the subject of many theories and speculations since Bang[1] first discovered the organism growing in a dense zone about 2 mm. below the surface of a tube of semisolid medium. These have all served their purpose well. The reduced oxygen tension theory, as applied to Bact. abortus, which has served to explain why so many of the strict anaerobic parasites may be grown on artificial media, had its origin in the work of Bang, and found its echo in many investigators who later attempted its cultivation. The reduced oxygen theory seemed to be fully substantiated when Nowak[2] demonstrated that visible growth could be obtained from infective material by placing the cultured material in closed containers with cultures of Bacillus subtilis. He was impressed with the idea that B. subtilis grew rapidly and would use up a considerable quantity of the oxygen in the container and thus create the proper reduced oxygen tension for the growth of Bact. abortus.

The sealed tube method was created by Preisz[3] with the same idea in mind. For a number of years all previously described methods and culture mediums were used in this laboratory in culture studies of the abortion disease. None of the existing methods was ever highly satisfactory. So in 1919, we began a study of the physiology of the growth of Bact. abortus and at the same time tried to determine the physiologic condition peculiar to the pregnant uterus and the udder which would account for the selection of these two organs as a place for the development of this organism. Our findings were the same in both the animal and on artificial culture mediums.[4] Our studies revealed that B. subtilis in its rapid growth and respiration produced an increased carbon

[1] Ztschr. . Thiermed., 1897, 1, p. 241.
[2] Ann. de l'Inst. Pasteur, 1908, 22, p. 541.
[3] Centralbl. f. Bakteriol., I, O., 1902, 33, p. 133.
[4] Cornell Veterinarian, 1921, 11, p. 210.

dioxide tension and made possible the rapid primary growth of Bact. abortus when placed in the same container; that when Bact. abortus was seeded in sufficient quantity on solid medium and the tube sealed and placed at a suitable temperature, sufficient carbon dioxide would in time be produced through respiration to bring about a visible growth, that is, when the proper carbon dioxide tension was reached. The volume of carbon dioxide in a closed container or tube when properly measured was found to average 10%. The volume of carbon dioxide in the fluids of the udder and the pregnant uterus was also found to average about 10%. This amount of carbon dioxide was then introduced artificially into jars in which mediums seeded with infective stomach exudate had been placed. To our surprise, a heavy growth of Bact. abortus was obtained after 48 to 72 hours of incubation at 37 C. Excellent growth was obtained with recently isolated strains of Bact. abortus after 24 hours under the same conditions. Further studies showed that if contaminating organisms were also present or a large number of cultures were placed in the jar, 5% of carbon dioxide introduced artificially was sufficient. The living organisms present rapidly produce sufficient carbon dioxide through respiration to bring about the necessary tension.

That newly isolated strains of Bact. abortus will grow in as low as 1% of carbon dioxide was shown by Smith.[5] We have not, however, succeeded in producing a visible growth of the organism directly from infective material in such a low percentage of carbon dioxide, if the original tension was maintained by removing every 24 hours the excess of carbon dioxide produced by the respiration of the growing organisms.

There appears to be no question at the present time concerning the necessity of an increased carbon dioxide for the primary growth of Bact. abortus, since the original findings have been confirmed by Fitch,[6] Traum,[7] Buck[8] and by Smith.[5].

Carbon dioxide was at one time thought to furnish a means for the primary cultivation of gonococcus,[9] and meningococcus[9a] mainly by either lowering the oxygen tension of the atmosphere or influencing the reaction of the medium as believed by Gates,[10] and Kohman[9] in the case of meningococcus. Later, however, Torrey and Buckwell[11] demonstrated conclusively that an increase in the atmospheric carbon dioxide had nothing to do with the primary isolation of gonococcus.

The study of the principle or principles which govern the rapid growth of Bact. abortus on culture mediums under an increased carbon dioxide tension have been continued by us, both with infective milk and material from infected fetuses. These studies have led to many interesting observations.

The ability of a freshly isolated strain of Bact. abortus to multiply was demonstrated by Smith[5] to be a function of the number of living organisms transplanted to a given culture medium. But the growth

[5] J. Exper. Med., 1924, 52, p. 501.
[6] J. Infect. Dis., 1922, 31, p. 233.
[7] Univ Calif. Agric. Exper. Sta. Bull. 353, 1923.
[8] J. Agric. Research, 1925, 29, p. 585.
[9] Chapin, C. W.: J. Infect. Dis., 1918, 23, p. 342. Kohman, E. F.: J. Bact., 1919, 4, p. 571. Herrold, R. D.: J. Am. M. A., 1920, 74, p. 1716. Rockwell, G. E., and McKahnn, C. F.: J. Infect. Dis., 1921, 28, p. 249.
[9a] Cohen, M. B., and Fleming, J. S.: J. Infect. Dis., 1918, 23, p. 337.
[10] J. Exper. Med., 1919, 29, p. 321.
[11] J. Infect. Dis., 1922, 31, p. 125.

function appears to be governed by three biochemical behaviors or reactions for which the organism itself is responsible. Their occurrence and the extent to which they occur are due, principally to the number of living bacteria on or transferred to the solid culture medium. The reactions involved are the production of carbon dioxide mainly through respiration, and the formation of ammonium magnesium phosphate and hydrogen sulphide by the bacterial decomposition of certain amino acids. These biochemical phenomena occur when the organism is grown on either meat or beef liver infusion agar, but the degree of the reactions is more pronounced on the latter due to the fact that the medium contains an abundance of one of the most necessary food constituents for growth, namely, an amino acid compound containing sulphur, presumably cystine or taurine.

The respired carbon dioxide plays two rôles in the growth of the freshly isolated organism, one is the increasing of the carbon dioxide tension of the surrounding air; the other tends to change the P_H of the medium to the point favorable for growth. The necessity for an increased carbon dioxide tension is transitory, and the amount required decreases with each successive transfer until the organism grows readily in open tubes requiring only the percentage of carbon dioxide contained in the air. If the medium employed in primary cultivation of the organism or of a recently isolated strain possesses an alkaline reaction, say P_H 7.2, a gradual decrease in the P_H may be observed if an indicator such as bromthymol blue be present. The first decrease will be noted on and for a slight depth under the surface at a point where the largest number of viable organisms is present. As the carbon dioxide tension increases from respiration, visible growth will appear on other parts of the surface less densely seeded. The color of the indicator in other parts of the medium will also change and gradually extend downward into the depth of the medium. By the time this will have occurred, a heavy growth of Bact. abortus will be visible on the surface of the sealed tube of medium.

When Bact. abortus in infective material or a newly isolated strain is planted on a culture medium, its immediate tendency is to change the carbon dioxide tension of the atmosphere to the tension to which it has been accustomed, and to derive energy from a suitable food in the medium for growth and reproduction. The proper food for this purpose appears to be, from our studies, an amino acid containing sulphur. So, in the decomposition of this amino acid, ammonia and hydrogen sulphide are liberated. The ammonia liberated tends to increase the P_H of the medium and actually does if the respired carbon

dioxide is not retained to neutralize its action. If this reaction does not occur, the ammonia unites with magnesium phosphate, a compound found in nearly all mediums made from meat, thus forming ammonium magnesium phosphate. This salt crystallizes out as visible crystals in the surface growth and just under the surface of the medium when the P_H of the surface growth and medium passes 7.0 or the neutral point. When the neutral point is reached hydrogen sulphide is no longer liberated as a free gas but is retained as sulphide of sodium, calcium, etc. The presence of the phosphate and sulphide compounds inhibits further growth of the organism. In other words, Bact. abortus in its effort to grow and multiply also at the same time is instrumental in bringing about changes which inhibit its growth and are partly responsible for its short span of life, under certain conditions.

The above changes may be observed if a slant of solid medium of an acid reaction, P_H 6.6, containing the indicator mentioned be seeded with a recently isolated strain of Bact. abortus, and sealed, or an aerobic strain and left unsealed, then incubated until a heavy growth is obtained. During the early multiplication of the seeded organisms on both the open tube inoculated with the aerobic strain, and the closed tube inoculated with the recently isolated strain, the hydrogen sulphide liberated may be detected by placing a piece of lead acetate paper in the tube beside the cotton plug. The recently isolated strain will be observed to be the more active of the two in the production of hydrogen sulphide. This is also true even though the tube be left unsealed. The indicator paper will begin to turn black in about two hours after the tube is placed in the incubator. As soon as the maximum growth is reached in the open tube culture, the reaction of the medium, as shown by the change in color of the indicator, will become alkaline, beginning in the growth and gradually extending downward into the medium. The liberation of hydrogen sulphide ceases when a P_H of 7 is reached. In a few hours, minute macroscopic crystals make their appearance, first in the growth and later under the surface of the slant. They gradually increase in size and in about seven days may become 1 mm. in width, and 1 to 3 mm. in length. Now if the sealed tube of the recently isolated strain is opened, the same changes will occur. Apparently the respired carbon dioxide is absorbed by the medium in the same proportion as is found in the air over the growth in the sealed tube. The constant acid reaction inhibits the formation and crystallization of the salt. The formation may also be inhibited by placing cultures in sealed jars in which part of the air has been replaced by carbon dioxide gas. The

growth activity of an old aerobic strain decreases as the formation of the salt increases, due to the fact that the respired carbon dioxide passes into the air very rapidly.

It is interesting to observe that the quantity of crystals formed varies in old laboratory strains, and the length of time that a culture will remain viable without transfer often depends upon the quantity of the crystals produced. We have three old laboratory strains from Europe in which the crystals are formed in great abundance, and, in order to keep these strains alive under aerobic conditions, it is necessary to transplant them every ten days. They will survive for weeks without transfer if the new growth is kept sealed to prevent the formation of the salt in question.

Identical changes, but of a different degree, have been observed in cultures of Brucella melitensis and paramelitensis. The formation of the crystals of ammonium magnesium phosphate is much more rapid and consequently the liberation of hydrogen sulphide reduced to a minimum. If, however, the inoculated tubes are kept sealed or are placed in a 5 to 10% carbon dioxide atmosphere, hydrogen sulphide will be liberated and may be detected with lead acetate paper. The difference in hydrogen sulphide production of Bact. abortus, and Br. melitensis and paramelitensis under aerobic cultivation serves as a very practical and rapid means of differentiating them when the variety to which a given culture belongs is unknown or undetermined. Our studies on the formation of ammonium magnesium phosphate and hydrogen sulphide by both Bact. abortus and Br. melitensis have been carried out in cooperation with O. B. Winter of the chemistry section and will be described more fully in another paper.

MEDIUMS AND METHODS FOR ISOLATING AND CULTIVATING BACTERIUM ABORTUS

Medium.—The infusion agar beef liver medium described by Stafseth [12] has been used in this laboratory with a considerable degree of success over all other mediums for isolating and cultivating Bact. abortus and Br. melitensis. The medium was prepared as follows:

Infusion: Fresh beef liver, free from fat, is ground in a meat chopper to a plastic mass. One pound of the liver and 500 cc. of tap water are placed in a container and mixed well. The container is covered and placed in flowing steam for twenty minutes. Remove the lid and stir with a glass rod in order to mix thoroughly so that all parts are reached by the heat. The heating is continued

12 Mich. Agric. Exper. Sta. Tech. Bull. 49, pt. 2, 1920.

in flowing steam for 1½ hours. Remove and filter through a wire screen. The infusion thus prepared may be placed in flasks and sterilized at 15 pounds pressure for 30 minutes, or made into liver medium at once.

To prepare 1 liter of liver infusion agar, measure out the following ingredients:

Washed agar	20 gm.	Peptone (Bacto.)	10 gm.
Tap water	500 cc.	Sodium chloride C.P.	5 gm.
Liver infusion	500 cc.		

Place all ingredients in a suitable container, cover and place in flowing steam for thirty minutes. Remove and cool to 60 C. Adjust the P_H at this time to 7. Add dissolved egg albumin to the extent of 1%. Mix well and place in flowing steam for 1½ hours. On removing the container from the steamer, one may note that the coagulated albumin has formed a firm clot at the top of the medium. The clot may be removed or the liquid slowly decanted. The medium still contains scattered clumps of albumin which may be removed by employing a filter made from a discarded pressure filter. Glass wool, previously washed with dilute acid to remove soluble alkali, is placed in the barrel of the filter. In the top is placed a thirty-mesh copper screen funnel for collecting large coagulated particles. Cotton or filter paper is not used in the preparation of this medium as it has been found that a considerable amount of the growth promoting properties of the medium are lost if passed through such material.

It is always necessary to determine the reaction of the medium after filtration as the P_H may be increased by soluble alkalies in the glass wool. The reaction is adjusted either with N/1 hydrochloric acid or N/1 sodium hydroxide.

Sterilize at 15 pounds pressure for thirty minutes. The reaction of the medium after sterilizing will usually drop to the proper P_H of 6.6.

The process of clarifying with egg albumin and filtration may be dispensed with if a Sharples centrifuge is available. This serves its purpose very efficiently and dispenses with much time and labor.

Methods of Cultivation.—When infective material is to be cultured for the presence of Bact. abortus, a prepared saturated aqueous solution of gentian violet is added to the liquified medium in a sufficient amount to dilute the dye solution 1 : 10,000. The presence of the dye inhibits the growth of the majority of gram-positive organisms, especially fast growing ones, but does not inhibit the growth of Bact. abortus. Colonies on this medium have a very characteristic appearance and may easily be distinguished from others.

Plates or tubes inoculated with infective material, are incubated at first aerobically at 37 C. for fifteen hours, and are then divided in equal numbers, one half incubated aerobically for ten days, and the other half placed in an atmosphere which has been replaced by 5 to 10% carbon dioxide and incubated for 72 hours.

When anaerobic cultivation is desired, Whitall Tatum museum jars are used. The tops of the jars are closed with a specially designed flat round metal top 3 mm. in thickness on which are sealed two metal one-

way cocks. The opening of one cock extending 6 mm. below the top to which is fastened a rubber or small glass tube running to the bottom of the jar in order to carry the introduced carbon dioxide to the bottom. The carbon dioxide is obtained from ordinary commercial tanks.

The methods used to detect the presence of Bact. abortus in the uterus, fetal membranes, fetal organs, milk, guinea-pig organs, human blood and urine have been derived from much study and experience not alone by us, but by other investigators as well. They are continually being improved as opportunities permit. A detailed description follows:

Uterus: If the nongravid uterus is to be examined for the presence of Bact. abortus, a fold of the cervix is first grasped by uterine forceps, retracted or left in normal position. A sterile metal catheter, 45 cm. by 6 mm., is then passed through the cervical canal. To the free end of the catheter is attached a piece of rubber tubing about 45 cm. in length which is in turn attached to a 250 cc. siphon flask containing 200 cc. of sterile physiological salt solution. The flask is inverted and held above the level of the uterus to allow the salt solution to flow in freely. A hand is now introduced into the rectum, the uterus palpated and massaged gently for about half a minute. The flask is now lowered to permit the fluid in the uterus to flow back into the flask. The uterine washing is taken to the laboratory and 0.05 or 0.1 cc. amounts spread over the surface of a gentian violet, liver agar plate in order to get some idea quantitatively as to the number of Bact. abortus present in the washing. The remaining fluid is centrifuged for $\frac{1}{2}$ hour at 4000 r. p. m., the sediment cultured as above and injected intra-abdominally into a guinea-pig. As a routine procedure, when seeking Bact. abortus, guinea-pigs are not killed and examined until after six weeks, regardless of the nature of the material injected.

When a parturient uterus is examined for evidence of infection, a sterile swab on the end of a 16 guage wire inclosed in a glass tube, 45 cm. by 6 mm., is introduced deeply into the uterus, removed, and the swab washed in 5 cc. of sterile salt solution and the solution cultured as previously described. The remaining amount is injected into a guinea-pig.

Fetal membranes: If the membranes are retained, two of the intact maternal-fetal placentae are removed at the peduncle and torn from the membranes. The excess liquid with which they are usually covered is removed by sterile gauze, and the chorionic and maternal villi separated. The exposed surfaces are now scraped with a sterile scalpel, the exudate obtained is cultured and suspended in sterile salt solution for injecting into a guinea-pig.

If the intact fetal membranes are obtained, they are laid open and spread out on a stone floor or table with the maternal side up. The examination of the placentae and interplacental areas for evidence of macroscopic changes is conducted according to the method described by Williams.[13] The debris and excess of blood are washed out by a slow flowing stream of water from a hose. The villi float in the running water thus aiding materially in the detection of evidence of disease. Placental areas and parts of areas showing what appears to be evidence of necrosis are removed, washed in five changes of sterile salt solution, ground in a mortar with sterile quartz sand and sufficient sterile salt solution added to make an emulsion. The emulsion is then cultured and a 2 cc. portion injected subcutaneously into a guinea-pig. This route is

[13] Cornell Veterinarian, 1925, 15, p. 255.

used in order to avoid a possible peritonitis and subsequent death of the pig from contaminating organisms. The animal inoculation technic described by Nelson[14] for isolating Bact. abortus from the uterine exudate and diseased placenta has not been so satisfactory in our work due to the loss of guinea-pigs from peritonitis within 24 to 48 hours after inoculation.

Fetus: In examining the aborted fetus for evidence of Bang's abortion disease, it is well to observe its physical appearance, the presence of a thin layer of brownish-yellow exudate sticking to a considerable area of the body, usually the ventral part, the color and consistency of the meconium adhering to the rectum and on exposing the viscera, the appearance of all organs. The macroscopic change observed in the appearance of the contents of the fourth stomach is almost certain evidence of the presence of Bact. abortus. If the fluid contents is found to be very viscous, of a straw or lemon yellow color and containing a considerable amount of suspended particles, this is unmistakable evidence of the presence of Bact. abortus. The writers have also observed that an invasion of the fetus by the small vibrio described by McFadyean and Stockman[15] and by Smith[16] will often produce changes in the contents of the stomach not unlike that caused by Bact. abortus. The presence of the vibrio may be easily detected by microscopic examination of stained smears of the stomach exudate. The latter procedure should be a routine one in the examination of the stomach exudate of all fetuses.

In culturing the various organs of the fetus for the presence of Bact. abortus about one square inch each of spleen, liver, kidney, and lung is removed with sterile instruments, the surfaces are incised in several places and smeared vigorously over the surface of several gentian violet, liver agar plates. The stomach exudate and heart blood are removed with a sterile pipet and placed in test tubes. Various amounts of these fluids are smeared over the surface of plates of the same medium. Meconium is removed from the large intestines or cecum with a sterile spatula and cultured in a similar manner. The small pieces of the various organs after culturing are ground with sterile quartz sand in a mortar. Sufficient sterile salt solution, 5 to 10 cc., is added for collecting the finely divided particles of tissue. The suspensions of the organs are injected intraabdominally into guinea-pigs in 2 cc. amounts. Two cc. of the stomach exudate are likewise injected into a guinea-pig.

Guinea-pig inoculation and examination: When Bact. abortus is sought for in any material through guinea-pig inoculations, the injections are given intraabdominally with the exception of necrotic fetal placentae, which is given subcutaneously. The pigs are always weighed before injection and at the time of necropsy. A loss of weight or no gain in weight is often times an indication of infection. Six weeks are allowed to elapse before necropsy. The presence of enlarged joints of the extremities is noted. The viscera and internal organs are exposed and all, including the lymph nodes external and internal, are examined for enlargement and evidences of macroscopic changes so characteristic of the disease in guinea-pigs. A blood sample is taken from the heart to determine the presence of agglutinins for Bact. abortus. The lungs, liver, spleen, kidneys, testicles, urine, and enlarged lymph nodes are cultured for Bact. abortus by removing pieces of each organ, clipping the cut surface in several places with a pair of sterile scissors and rubbing the cut surface over the surface of a gentian violet, liver agar plate. The plates are incubated

[14] J. Exper. Med., 1926, 43, p. 331.

[15] Report on Epizootic Abortion, Department Committee, Bur. Agriculture and Fisheries, pt. 3, 1913.

[16] J. Exper. Med., 1919, 30, p. 313.

for 15 hours at 37 C. and then placed in jars in which 5 to 10% of the air has been replaced by carbon dioxide. The inoculated plates are incubated in carbon dioxide for 72 hours at 37 C. and examined for colonies of Bact. abortus. If aerobic types of the organism are sought for, an additional set of plates is made from the same organs and held at 37 C. for seven days, daily examinations being made for suspicious colonies.

Human blood and urine: Since undulant fever in man due to Bact. abortus or Br. melitensis is becoming a diseases of increasing importance in this country and since this laboratory happened to be fortunate enough to have the opportunity to study several cases, it seems proper that the technic employed by us for culturing the organism from blood and urine be presented here.

For the blood culture a 5 to 10 cc. sample is drawn from the median cephalic vein with a sterile syringe or in a Kidel tube. If the blood has not clotted, ¼ cc. amounts are spread over the surface of six gentian violet liver agar plates and the remaining amount placed in 100 cc. of liver infusion broth. In case the blood has clotted as in a Kidel tube, the whole clot is removed, a piece severed from the whole and spread on the plates as above, and the remainder dropped into 100 cc. of the broth. Three of the plates are placed in carbon dioxide jars immediately and three incubated aerobically at 37 C. for ten days. The inoculated flasks of broth are placed in carbon dioxide jars immediately and incubated for at least ten days at 37 C. If the organism is present in considerable numbers, a growth will be noted in the bottom of the flask in the form of small white or grayish clumps at about the 72nd hour. If a sufficient amount of blood is available, incubation should be carried out aerobically as well as in carbon dioxide in order to determine if the infecting organism is an aerobic type. Newly isolated strains from humans appear to differ as to their carbon dioxide requirements after primary growth. Two strains which we have isolated required twelve transplantations before losing their carbon dioxide requirements, while one strain which we have just recently isolated grew well aerobically on the first seeding from the blood culture.

The presence of Bact. abortus or Br. melitensis in urine is not constant, and several samples may be taken and cultured before it is isolated. Our practice is to centrifuge a 100 cc. sample of urine at 4000 r. p. m. for thirty minutes and spread the sediment over several plates of media and incubate in the same manner as the blood sample.

Milk: The detection of the presence of Bact. abortus in apparently normal milk or colostrum through the methods first described by Schroeder and Cotton[17] and independently at about the same time by Smith and Fabyan[18] through guinea-pig inoculations, or by any other possible method has for many years been a problem of immense importance, especially with us. The carrier state or disease of the udder and its nature is considered to be one of the fundamental problems which confront those who are studying the abortion disease. It may be considered as being its primary focus and also its terminal focus. It appeared to us that, in order to arrive at the fundamental nature of the disease in the udder, a more rapid and quantitative means of measuring the presence of Bact. abortus was necessary. The fact that it could be cultured from the milk of an artificially infected cow as demonstrated by Evans,[19] cleared the way for its isolation from naturally infective milk.[20] This was

[17] Proc. Am. Vet. M. A., 1911, p. 442.
[18] Centralbl. f. Bakteriol., I, O., 1911, 61, p. 549.
[19] J. Infect. Dis., 1918, 23, p. 354.
[20] Huddleson, I. Forest: Mich. Agric. Exper. Sta., Tech. Bull. 49, pt. 4, 1920.

made possible through the use of a medium developed by Stafseth,[12] the proper atmospheric conditions to which the organism has adapted itself in the udder of the cow and the incorporation in the medium of a highly selective dye (gentian violet) which inhibits the growth of many organisms commonly found contaminating milk. The technic previously employed in which the centrifugated sediment of milk was utilized and compared with a 5 cc. sample of freshly drawn whole milk injected intraabdominally into a guinea-pig many times failed to check with positive guinea-pig findings. Further study of cultural methods has revealed the cause of the failure and has led not only to more accurate means of detecting its presence but of estimating the number present quantitatively and its relative position in different parts of the milk. The data representing our later studies follow:

Material for Study.—There have been utilized in these studies, cows from naturally infected herds which have aborted one or more times and which have constantly shown the organism in the milk from one or all quarters for one or more years. The amount of milk produced daily by a given infected cow varies with the degree of infection in the udder. Many of the cows produced as high as 25 pounds of milk per day, while others produced not more than one pound and even this amount gradually became less late in lactation.

Both morning and evening samples were utilized in the study, there being little or no difference in the results. When the samples were to be taken, the udder and teats were first moistened with water or a weak antiseptic solution and the moisture removed from the teats with a dry cloth. The first three streams of milk were always discarded before collecting the sample to be examined in a sterile test tube or flask. The samples were taken directly to the laboratory and the whole milk cultured at once, but not before vigorous shaking when a quantitative estimation of number of organisms present was desired. The milk was cultured by placing either 0.05 or 0.1 cc. on the surface of solidified gentian violet liver agar plates in triplicate, and then spread evenly over the surface of the plate by means of a sterile glass rod, with right angle bend, by rotating the plate in a horizontal plane.

That this method gives a fairly accurate estimation of the number of Bact. abortus in milk is shown in table 1. In this case the milk was drawn from the right rear quarter of a cow and the same amount cultured on each of 10 plates. The results shown represent one of several series of counts which we have made on many samples of whole milk from different cows. The difference in the total count appears to be no greater than what one would find in making ordinary plate counts of milk with a constant quantity of milk on a large number of plates.

The quantitative study of the presence of Bact. abortus in milk has revealed a surprising variation in the counts from different cows and a marked similarity in the count of milk from the same cow taken at various intervals both in the morning and evening. Total counts from

different cows vary from 5 colonies to 10,000 colonies per cc. of whole milk. The quantity of milk produced does not appear to be a constant factor in the quantity of organisms found. For example, a cow whose milk showed a count of 10,000 colonies per cc. was constantly producing 25 pounds a day. In a given cow, however, the total count appears to increase toward the end of lactation or as she is being dried off for a subsequent parturition. At this time the milk is densely populated with Bact. abortus.

Position of Bact. Abortus in Milk.—It is desirable to know the position Bact. abortus takes in milk left standing, or centrifugated. In view of the fact that freshly drawn milk contains suspended fat globules and often a high cellular content, and that these separate into a distinct

TABLE 1

The Quantitative Estimation of Bact. Abortus in Infective Whole Milk

Plates	Number of Colonies per 0.1 Cc. of Milk
1	98
2	90
3	114
4	115
5	65
6	103
7	57
8	108
9	175
10	54
Average	97.9

zone in milk left standing or centrifugated, it appeared highly desirable to know what position Bact. abortus takes. This determination was carried out by selecting samples of milk from cows in which the organism had been previously detected by cultural methods.

The freshly drawn samples in 15 cc. amounts were placed in test tubes in which the bottoms had been drawn out to a fine capillary point of about 1 mm. in diameter, and sealed by heat. The samples were placed in an icebox at 5 C. for 24 hours. At this time the separated cream was removed with a pipette and 0.1 cc. amounts cultured in triplicate. The sealed end was broken and the first bottom cubic centimeter collected. After mixing thoroughly, 0.1 cc. amounts were cultured in triplicate and the remaining 0.7 cc. injected intraabdominally into a guinea-pig in order to make certain of the presence of Bact. abortus as previous preliminary experiments had often failed to show the organism in the bottom gravity separated milk. The plates were incubated aerobically for 15 hours and then placed in jars containing 5% carbon dioxide for 72 hours. The guinea-pigs were held six weeks before necropsy.

The results of an experiment of the above nature on nine samples of milk are set forth in table 2. The colony counts on the whole milk

samples immediately after collection, of the gravity cream and bottom portions are shown. In comparing the counts it is quite evident that the rising butter fat and tissue cells (polymorphonuclear leukocytes and lymphocytes), which also rise with the fat, bring up the majority of Bact. abortus in the milk. It was interesting to note the small amount of sediment which actually collected at the capillary end of the tubes. The volume varied from 1 to 3 mm. in length at the tips of the tubes. The quantity of tissue cells in cream separated from milk by gravity and by centrifugation was first observed by Prescott and Breed.[21] They found that only 2.5 to 50% of the total tissue cells in whole milk are found in milk sediment and a greater percentage rise with the cream. Since the tissue cells of the type found in milk have approximately the

TABLE 2

The Quantitative Position of Bact. Abortus in Infective Milk After Standing 24 Hours at Ice Box Temperature

Sample	Number of Colonies in 0.1 Cc.		
	Cream	Bottom Milk*	Whole Milk
1	520	6	40
2	450	2	34
3	140	2	20
4	90	1	14
5	100	2	30
6	280	3	35
7	140	6	30
8	89	2	15
9	160	3	20

* Guinea-pig inoculations with bottom milk produced no characteristic tissue changes.

same specific gravity as butter fat it would not appear remarkable to find the cells rising with the fat. Apparently, single specimens and clumps of the abortion organism cling to the cellular elements and fat globules and are brought to the top of the column of milk. It is, there-fore, evident that the gravity cream or butter fat is the logical portion of milk to examine in the detection of the presence of Bact. abortus qualitatively. Furthermore, the organisms are concentrated at the top very quickly as we have found that after milk has stood for only a period of two hours the number recovered from the cream layer is much greater than the number recovered from the whole milk.

The failure to detect the presence of Bact. abortus in the portions of bottom milk by means of guinea-pig inoculations was very much of a surprise to us since Hagan[22] had demonstrated that approximately nine

[21] J. Infect. Dis., 1910, 7, p. 132.
[22] J. Exper. Med., 1922, 36, p. 697.

organisms were sufficient to produce tissue changes characteristic of the abortion disease in such animals through inoculation. From the approximate number obtained by culturing it is quite evident that these pigs received more than a total of nine organisms. Guinea-pigs receiving samples 4, 8 and 9 showed agglutinins present in a 1 : 50 dilution of the blood serum. This alone is evidence that Bact. abortus had been present in the pigs, but not in sufficient numbers to establish itself and produce macroscopic change in the organs. Whole milk samples in 5 cc. quantities from all of the cows have constantly produced the characteristic abortion disease in pigs. From these results it is evident that gravity milk minus the cream is unsuitable for determining the presence of Bact. abortus through guinea-pig inoculations.

Further consideration was given to this phase of the problem by comparing the quantitative position of the organism in the cream, middle milk and sediment, in both gravity and centrifugated specimens.

TABLE 3

The Quantitative Estimation of Bact. Abortus in Centrifugated Cream and Sediment, and in Gravity Cream and Sediment

Material	Cc.	Number of Colonies	
		Sample 8	Sample 84
Whole milk	0.1	58	90
Centrifugated cream	0.1 gm.	207	77
Centrifugated middle milk	0.1	3	3
Centrifugated sediment	0.1 gm.	251	18
Gravity cream	0.1	552	209
Gravity middle milk	0.1	4	0
Gravity sediment	0.1	18	1

For the gravity estimation the procedure previously described was employed. For the centrifuge estimation a 15 cc. sample of the same milk was placed in a conical tube and centrifuged at 4000 r. p. m. for 30 minutes. The butter fat was removed with a sterile spatula and 0.1 gram portions cultured in triplicate, 0.1 cc. portions of the middle milk and 0.1 Gm. portions of the sediment was cultured in a similar manner. The inoculated plates were incubated as previously described. Table 3 presents the comparative colony counts from the average of each of three plates. Here again is revealed the greatly increased volume of organisms in the gravity cream over any other portion of the milk regardless of centrifugation. In the sample from cow 8 the centrifugated sediment count is greater than the centrifugated cream. While the sample from cow 84 is the reverse. It is also quite evident that there is very little difference in the count made from middle milk and gravity sediment. Both being very small in comparison to the cream count.

A knowledge of the viability of Bact. abortus in infective milk is important since it is often impossible to examine milk immediately after collection and, too, milk may be shipped some distance and many hours may intervene before it arrives at the examining laboratory.

For this determination samples were collected from several infected cows, cultured immediately in triplicate and placed in an icebox at 15 C. for various lengths of time before culturing was done. The sample were always shaken vigorously for one minute before culturing in order to break up the cream layer. Table 4 illustrates one of several experiments of this nature which has been carried out. The results indicate that a sample of milk may be held for at least eight days at a low temperature without affecting the total count of organisms present, while at the eleventh day as one might expect, a considerable decrease in the total count occurs. The holding of milk for a longer period than 24 or 48 hours, of course, is impractical unless considerable aseptic precaution is exercised in its collection and proper cooling.

TABLE 4

The Viability of Bact. Abortus in Infective Milk Held at Ice Box Temperature

Sample	Number Days in Icebox	Colonies per 0.1 Cc. Whole Milk
44	0	40
	8	44
	11	10
8	0	90
	3	95
	90	0
84	0	36
	38	5
	60	0

TABLE 5

The Effect of Aerobic Incubation on the Primary Isolation of Bact. Abortus

Number of Hours at 37 C. Before Placing in CO_2	Number of Colonies per 0.1 Cc. Whole Milk
0	348
24	226
48	139
72	50
96	34
120	22
144	2

Another factor in the cultivation of Bact. abortus from milk is the proper length of time to hold the inoculated plates aerobically in the incubator before placing in jars containing carbon dioxide. The aerobic incubation is often carried out to determine the presence of aerobic types of Bact. abortus and other aerobic organisms. The effect of aerobic incubation of inoculated plates is illustrated in table 5.

This experiment was carried out by inoculating each of 21 plates with 0.1 cc. of the same sample of whole milk. Three plates were placed in 10% carbon dioxide immediately and the remaining number in a moist air 37 C. incubator. Every 24 hours three plates were removed and placed in 10% carbon dioxide. The jars were opened at the end of 72 hours and the number of colonies counted

on each plate. The table shows the average count of the three plates of each series. The results indicate that the total colony count decreases in a steady falling curve as the number of hours the plates held aerobically increases. In other words, if the maximum colony count is to be obtained on a given sample of milk, the inoculated plates must be placed in carbon dioxide before the end of 24 hours.

DISCUSSION

Our studies show that in the course of the growth activity of Bact. abortus on culture mediums, three biochemical reactions may occur, depending upon whether the freshly seeded culture is sealed or open. The reactions occurring are: the production of respired carbon dioxide which serves to increase the atmospheric tension and to balance the reaction of the medium to the point most favorable to the growth activity of the organism; the production of hydrogen sulphide or sulphur compounds in case the medium remains or becomes alkaline in reaction, the hydrogen sulphide being split off from an amino acid containing sulphur by bacterial decomposition; the formation of ammonium magnesium phosphate in an alkaline medium when ammonia split off from an amino acid comes in contact with magnesium phosphate. The two gaseous compounds are probably derived from cystine or taurine.

The increased carbon dioxide tension which is so peculiar to the primary isolation and cultivation of Bact. abortus, and which may be dispensed with after a certain number of transfers, is more or less what might be termed a passive physiologic phenomenon. The fact that this change occurs in such a comparatively short time indicates that Bact. abortus was originally a true aerobe, that is, until successive passages through its host, the bovine, changed its atmospheric requirements to that of the organs of the host where it had also found the necessary food requirements for growth and multiplication.

Comparing Bact. abortus with Br. melitensis as respects host and organs parasitized, we find the latter selecting a closely related species, the caprine, and the same organs, namely the udder and pregnant uterus. Yet when artificially cultivated from these organs, Br. melitensis has never been reported as requiring an increased carbon dioxide tension. The question now arises, is the carbon dioxide tension of pregnant uterus and udder of the goat distinctly different from that of the cow, and has Bact. abortus become a variety of Br. melitensis, by continued passage through the cow? Experiments conducted by Schroeder and by us indicate that Br. melitensis does not establish itself in the udder of the cow. He did not succeed in isolating it from the udders of two cows in which abortions were induced by intravenous inoculation. We suc-

ceeded in isolating it from the two quarters of a cow for only three weeks after inducing an abortion in a heifer in the same manner.

The methods for determining the presence of Bact. abortus in infective material are presented with the idea of arriving at a basic comparison and interpretation of results with those of others who are engaged in abortion disease studies. As is often the case, workers interpret their findings on similar problems differently, due to the fact that there are so many variations in methods. This unquestionably leads to confusion and makes the solution of a given problem difficult.

In reference to the detection of the presence of Bact. abortus in milk it is evident that there is quite a variation in the methods employed. In fact there seems to have been very little work done in the way of studying the degree of the disease in the udder on the basis of numbers. Furthermore, it would appear from the results of our studies on the isolation of Bact. abortus from milk that those who have heretofore discarded the cream layer have also discarded the majority of the organisms they are seeking and thus reduced greatly the possibility of recovering the organism through guinea-pig inoculations. The failure of the guinea-pigs to become infected with the gravity sediment and bottom milk of the samples recorded in table 2, is ample proof that the sediment, even though it contains a few organisms is not the desirable part of milk to use in order to establish the presence or absence of Bact. abortus.

In this connection Fitch and Lubbehusen[23] made some very extensive studies on the comparative value of whole milk and milk sediment for the presence of Bact. abortus through guinea-pig inoculations. Relatively large samples of milk were collected from each cow studied and allowed to stand in ice box temperature for 24 hours to permit the separation of the cream. The cream was discarded and the centrifuged sediment from a 200 cc. sample of the remaining milk was used for inoculation purposes. The amount of whole milk used for guinea-pig inoculation was 5 cc., and of the sediment 3 to 5 cc. The detection of Bact. abortus by the two methods did not agree very closely. In eleven out of a possible 37, the organism was recovered from the whole milk and not from the sediment, and in 16 samples the organism was recovered from the sediment and not from the whole milk. The fact that the majority of the organisms are brought to the top with the rising fat globules and body cells would serve to explain part of the discrepancies between the methods.

While the guinea-pig inoculation method is admirably adapted for the detection of Bact. abortus it is too slow a procedure to use in studying the course of the disease in the udder of one or many cows. And,

[23] Cornell Veterinarian, 1926, 16, p. 46.

too, no quantitative determination can be made by following this procedure.

We are convinced that the culturing of the cream layer of milk and at the same time observing the necessary precautions as regards time of holding sample after collection, and primary incubation of inoculated plates, is just as efficient for detecting the presence of Bact. abortus as guinea-pig inoculation methods.

The culture method lends itself admirably to the study of the carrier state in one animal or several. Further, the method can easily be applied to the routine examination of market milk either qualitatively or quantitatively. The guinea-pig inoculation method is entirely out of the question as a routine procedure for detecting the presence of Bact. abortus in milk in public health laboratories.

SUMMARY

The growth activity of Bact. abortus is governed by an increased carbon dioxide tension, by the liberation of hydrogen sulphide and the formation of ammonium magnesium phosphate; the production of the latter two substances being controlled by an abundance of carbon dioxide and the reaction of the culture medium.

It is pointed out that there is a biochemical reaction which serves to distinguish Bact. abortus from Br. melitensis, that is, in the formation of ammonium magnesium phosphate and liberation of hydrogen sulphide.

Bact. abortus may be measured quantitatively in infective milk with a high degree of accuracy by employing, as a culture medium, gentian violet, beef liver infusion agar, and incubating in an atmosphere containing from 5 to 10% carbon dioxide.

In the gravity separation of cream the rising fat globules and body cells carry with them the majority of the abortion bacteria in milk.

The culturing of the gravity cream layer of milk is just as efficient for determining the presence of Bact. abortus in milk as are guinea-pig inoculation methods.

Bact. abortus does not appear to multiply in milk held at ice box temperatures and decreases in numbers rapidly when held longer than eight days.

The number of colonies of Bact. abortus which develop on plated culture medium, decreases as the length of time they are held aerobically at 37 C. increases.

EFFECT OF TYPE 1 PNEUMOCOCCUS CULTURE BROTH ON THE PROTECTIVE ACTION OF TYPE 1 ANTISERUM

G. M. SICKLES

From the Division of Laboratories and Research, New York State Department of Health, Albany

Many theories have been suggested concerning the protective mechanism against the pneumococcus. Foá and Carbone,[1] Kruse and Pansini[2] and Emmerich and Fawitzky[3] emphasized the bactericidal action of the serum. Issaeff[4] concluded that phagocytosis played a very important part in immunity against pneumococci. Mosny[5] believed immunity to be due to the antitoxic property of blood serum as did Klemperer and Klemperer[6] and Tizzoni and Panichi.[7] No true toxin was demonstrated by them, however.

Rosenow,[8] Cole,[9] Solis-Cohen, Weiss, and Kolmer,[10] Weiss[11] and others have reported the presence of toxic substances in extract of pneumococci, but have made no reference to the presence of the toxin in culture broth in which pneumococci are actively growing. On the contrary, Wadsworth[12] and Chesney and Hodges[13] report no toxic effects after the injection of pneumococcus culture filtrate. More recently, Olson[14] has produced a pneumococcus filtrate which, when injected intraperitoneally, gives rise to pneumonia in animals and is neutralized by an antitoxic serum prepared by Larson.[15]

These results bear out the report of Wadsworth[16] that the culture fluid of the pneumococcus, after growth for 24 hours, contains substances capable of inciting an immunity in the tissues which will so neutralize the products of the pneumococci present in the tissue that they will give rise to few or no signs of disease. Furthermore, "this action and reaction between soluble pneumococcus poison and the immunity suggests immediately the toxin-antitoxin reaction of diphtheria. The two are doubtless similar, if not identical in nature: but the toxin of diphtheria, although unknown chemically, is a powerful poison that is fatal for animals, and this poison may be titrated accurately against its antitoxin, whereas the soluble substances of the pneumococci are not fatal and are recognized only by the immunity incited by their presence."

[1] Gazz. med. di Torino, 1891, 42, p. 337.
[2] Ztschr. f. Hyg., 1892, 11, p. 279.
[3] München med. Wchnschr., 1891, 38, p. 554.
[4] Ann. de l'Inst. Pasteur, 1893, 7, p. 260.
[5] Arch. de méd. expér. et d'anat, path., 1892, 4, p. 195.
[6] Berlin. klin. Wchnschr., 1891, 28, pp. 833, 869.
[7] Centralbl. f. Bakteriol., Ref. I, 1905, 36, p. 25.
[8] J. Infect. Dis., 1911, 9, p. 190.
[9] J. Exper. Med., 1912, 16, p. 644.
[10] J. Infect. Dis., 1918, 22, p. 476.
[11] J. Med. Research, 1918-19, 39, p. 103.
[12] J. Exper. Med., 1912, 16, p. 54.
[13] Bull. Johns Hopkins Hosp., 1922, 33, p. 425.
[14] Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 295.
[15] Ibid., 23, p. 497.
[16] J. Exper. Med., 1912, 16, p. 78.

These observations suggested that there might be present in pneumococcus culture broth a substance which would neutralize the protective action of the immune serum when tested in proper dosage in a manner analogous to that of diphtheria toxin and antitoxin.

Tests were made in mice, using the following technic.

Organisms and culture broth of the laboratory standard type 1 pneumococcus, a very virulent strain, of which 0.00000001 cc. kills mice regularly, were used in most of the tests. In the preliminary experiments, the culture was centrifugalized, and toluene was added (1:10) to the supernatant culture broth and to the uninoculated broth control. For the remaining tests, culture broth filtered through a Berkefeld filter was used without preservative. The culture broth and diluted serum were mixed and incubated in a water bath at 37.5 C. for three hours. A separate bottle was used for each mouse. After incubation, the organisms were added, and the mixture was allowed to stand two minutes before injection (table 1).

TABLE 1

EFFECT OF TYPE 1 CULTURE BROTH ON THE PROTECTIVE ACTION OF TYPE 1 SERUM

Type 1 Serum, 0.001 Cc. Incubated 3 Hours at 37.5 C. with	Type 1 Organisms Added 2 Minutes Before Inoculation, Cc.	Effect of Inoculation on Mice, Hours Survival
Culture broth, 0.5 cc. Virulent type 1, 18 hour growth....................	0.001	19 26 26
	0.0001	19 19 38
Uninoculated broth	0.001	41 Living at 10 days Living at 10 days
	0.0001	17 Living at 10 days Living at 10 days

There was, apparently, a loss of protection in serum exposed to pneumococcus culture broth.

Method of Inoculation.—In one experiment the organisms were injected ½ hour after the serum, and in another, the serum, organisms, and culture broth were injected together without previous incubation of the culture broth and serum. Similar results were obtained in each case. The culture broth lessened the protective action of the serum, whereas uninoculated broth did not affect it. The method of inoculation, therefore, does not apparently influence the result.

Age of Broth.—According to tests, one and 21 days after filtration, the substance in the culture broth is unstable, for the effect on the protective action of type 1 serum had apparently diminished greatly during the 21 days. There was no preservative in the culture broth tested. The precipitation reaction with type 1 serum was still marked.

Effect of Heat.—Portions of the culture broth were tested after heating in a water bath at 45 C., 55 C., and 75 C. for thirty minutes, and for five minutes at boiling temperature (table 2). The action of the substance on the protective power of the serum was reduced slightly by heating for 30 minutes at 75 C. and by boiling for five minutes, but was not destroyed. All of the samples, after heating, gave a well-marked precipitation reaction with type 1 serum.

Amounts of Culture Broth.—Amounts of culture broth, from 0.1 to 0.5 cc., were tested with the same amount of serum. Little difference

TABLE 2

EFFECT OF HEAT ON SUBSTANCE IN TYPE 1 CULTURE BROTH

Type 1 Serum, 0.0066 Cc. Combined for 3 Hours at 37.5 C. with	Injection of Type 1 Organisms 0.00001Cc. Effect on Mice, Hours Survival
Culture broth 0.5 cc. Virulent type 1, 18 hour growth	
Unheated	41
	48
	48
Heated, 45 C. 30 minutes	41
	55
	67
Heated, 55 C. 30 minutes	41
	41
	48
Heated, 75 C. 30 minutes	65
	65
	Living at 10 days
Heated, boiling, 5 minutes	47
	47
	Living at 10 days
Uninoculated broth	3 mice, living at 10 days

was noted in the results when varying amounts were used. The mice which received 0.1 cc. died as soon as those receiving 0.5 cc. The experiment, however, is not conclusive since one of the controls also died.

Type Specificity.—Culture broths from the laboratory standard type 2 and type 3 strains were exposed with type 1 serum, and from the few experiments made, the substance appeared to be not completely type specific (table 3).

Presence in Type 3 Culture Broth.—One experiment only was done with type 3 culture broth and type 3 serum. Because of the low protective titer of the type 3 serum, it was used undiluted, and a heavy precipitate formed immediately when the type 3 culture broth was added. Each bottle was washed out with 0.5 cc. of uninoculated broth after the test material was withdrawn, and the broth used in washing was injected

into the peritoneal cavity of the mouse. Results suggested that a substance inhibitory to the protective action of the antiserum was present in type 3 culture broth.

Actively Growing and Older Cultures.—Cultures of type 1 were removed from the incubator after 4, 8, 12 and 18 hours, and 11 days, were filtered, and the culture fluid tested in mice in the usual manner (table 4). There was, apparently, very little of the substance present

TABLE 3

Specificity of the Substance in Pneumococcus Culture Broths

Type 1 Serum, 0.0066 Cc., Exposed 3 Hours at 37.5 C., with Culture Broth, 0.5 Cc., from	Injection of Type 1 Organisms	
	Cc.	Effect on Mice, Hours Survival
18 Hour Growth		(S = Living at 10 days)
Type 1	0.00001	41
		65
		70
	0.000001	66
		42
	0.0000001	90
		S
Type 2	0.000001	66
		S
	0.0000001	S
		S
Type 3	0.00001	43
		67
		S
	0.000001	S
		S
	0.0000001	S
		S
Uninoculated broth	0.00001	S
		S
		S
	0.000001	S
		S
	0.0000001	S
		S

in 4 hours, a small amount in 8 hours, and more after twelve hours. After eighteen hours' growth, the substance did not appear to increase to any extent.

Attenuated Cultures.—An attenuated form of the same type 1 strain, 1 cc. of which did not kill a mouse, was used (table 5). The 18 hour culture broth had a slight effect on the serum, and there appeared to be some substance in the older culture broth.

A Different Strain.—The culture broth of a different type 1 strain, obtained originally from the Hygienic Laboratory, of which 0.000001 cc.

TABLE 4

PRESENCE OF THE SUBSTANCE IN ACTIVELY GROWING CULTURES AND IN OLDER CULTURES

Type 1 Serum Combined, 3 Hours at 37.5 C. with Culture Broth, 0.5 Cc., from	Injection of Type 1 Organisms	
	Cc.	Effect on Mice, Hours Survival (S = Living at 10 days)
Serum 0.001 cc. Type 1,		
4 hour	0.00001	S, 3 mice
4 hour	0.000001	S, 3 mice
8 hour	0.00001	48 42 S
8 hour	0.000001	64 S S
12 hour	0.00001	56 42 S
12 hour	0.000001	88 54 S
Uninoculated broth	0.00001	8½ days S S
Uninoculated broth	0.000001	S, 3 mice
Serum 0.0066 cc. Type 1,		
4 hour	0.000001	S S
4 hour	0.0000001	S S
8 hour	0.000001	90 S
8 hour	0.0000001	S S
12 hour	0.000001	25 25
12 hour	0.0000001	42 S
Uninoculated broth	0.000001	37 S
Uninoculated broth	0.0000001	S S
Type 1,		
18 hour	0.00001	27 43 43
18 hour	0.000001	43 43 81
Uninoculated broth	0.00001	S, 3 mice
Uninoculated broth	0.000001	S, 3 mice
Type 1,		
11 day	0.00001	43 50 91
11 day	0.000001	67 81 S

killed a mouse in 48 hours, was tested with type 1 serum. The substance was present in the culture broth of this less virulent strain.

Effect on the Laboratory Standard Serum and on Rabbit Serum.— In most of the previous tests, the same serum, a horse antiserum for type 1, was used. Tests were made in which the culture broth was combined with another serum used as the laboratory standard since 1919,

TABLE 5

Production of the Substance by Attenuated Cultures

Type 1 Serum, 0.001 Cc., 3 Hours at 37.5 C., with Culture Broth, 0.5 Cc., from	Injection of Type 1 Organisms	
	Cc.	Effect on Mice. Hours Survival (S = Living at 10 days)
Type 1		
8 day, attenuated........................	0.001	25 18 25
8 day, attenuated........................	0.0001	4+ days 42 42
18 hour, virulent........................	0.001	19 26 26
18 hour, virulent........................	0.0001	19 19 38
Uninoculated	0.001	41 S S
Uninoculated	0.0001	17 S S
Type 1		
18 hour, attenuated.....................	0.000001	66 90 S
18 day, attenuated......................	0.000001	80 44 44
18 hour, virulent........................	0.000001	45 45 45
Uninoculated	0.000001	S S 89

and with a serum from a rabbit under immunization for $3\frac{1}{2}$ months with a strain of the first used type 1 organism, virulent for rabbits. The results were approximately the same with all antiserums.

Phagocytosis in Vivo.—Three mice were inoculated in the usual manner with serum, culture broth, and organisms; and three control mice with serum, uninoculated broth, and organisms. At the end of 4, 20, and 28 hours, smears of exudate withdrawn from the peritoneal cavity of a test mouse, and of a control mouse, were stained by Wright, Hiss, and Gram methods.

At the end of four hours, the smear from the test mouse with the pneumococcus culture broth showed leukocytes but no organisms. In the control mouse, with the uninoculated broth, there were many leukocytes, a few intracellular pneumococci, and no free organisms. After 20 hours, the exudate from a test animal contained many encapsulated extracellular, no intracellular pneumococci. No exudate was

TABLE 6

EFFECT OF EXPOSURE AT AN ELEVATED TEMPERATURE

Type 1 Serum, 0.0066 Cc., 3 Hours in Contact with Culture Broth, 0.5 Cc., from		Injection of Type 1 Organisms	
		Cc.	Effect on Mice, Hours Survival (S = Living at 10 days)
Incubation at 37.5 C.	Type 1; virulent 18 hour	0.00001	27
			43
			43
	18 hour	0.000001	43
			43
			81
	11 days.................	0.00001	43 ·
			50
			91
	11 day	0.000001	67
			81
			S
	Uninoculated	0.00001	S
			S
			S
	Uninoculated	0.000001	S
			S
			S
Incubation at 40.5 to 41 C.	Type 1, virulent 18 hour	0.00001	40
			40
			S
	18 hour	0.000001	40
			S
			S
	11 day	0.00001	4+ day
			S
			S
	11 day	0.000001	S
			S
			S
	Uninoculated	0.00001	S
			S
			S
	Uninoculated	0.000001	S
			S
			S

obtained from the control animal. In 28 hours, there were many encapsulated pneumococci in the exudate from the test mouse. Of one hundred leukocytes counted, 1 contained twelve pneumococci; 99, no pneumococci. In the exudate from the control mouse, there were no pneumococci and no polymorphonuclear leukocytes. All three of the test animals died; the three control animals lived.

Contact with Serum at an Elevated Temperature.—Eighteen hour and 11 day culture broths were exposed with the serum for three hours at 40.5 to 41 C. The effect of these mixtures on the protective antiserum action was considerably less than that of the control mixtures incubated at 37.5 C. (table 6). This decreased inhibitory effect was

particularly noticeable with the older culture broth. In the work of Felton and Bailey [17] on the biologic significance of the soluble specific substance of pneumococci, which was published while these experiments were in progress, the possibility of dissociation and liberation of the protective protein by heat is discussed. That the results are due to the effect of heat on the substance itself is not likely since it is apparently able to withstand higher temperatures before contact with the serum without changing its action on the protective power of serum added later.

Effect of Growth of Pneumococci.—Serum diluted 1 : 30 with pneumococcus broth was inoculated with a loopful of the type 1 strain from a blood slant to keep the volume the same and was incubated for 18 hours. Uninoculated diluted serum was incubated at the same time, and both were filtered through Berkefeld V filters. The results were practically the same as with the culture broth serum mixtures. The uninoculated control serum protected against the test doses used; the culture serum did not.

Precipitation tests made with the diluted serum (both the inoculated and the uninoculated) against the supernatant fluid after centrifugalization of a type 1 broth culture, were negative. With the undiluted pneumococcus antiserum a strong reaction was obtained with the culture serum; none, with the uninoculated control serum.

SUMMARY

There is present in type 1 pneumococcus culture broth a substance in the presence of which the protective action of type 1 pneumococcus serum is diminished.

The substance is relatively thermostable, deteriorates with age, and is not absolutely type specific.

It is not present in very young cultures in a sufficient amount to be detected by the method used, but it begins to appear about the 8th hour of incubation.

When the pneumococcus culture broth and antiserum are exposed together to a temperature between 40.5 and 41 C. the inhibiting effect of the broth on the protective action of the serum is considerably diminished.

When type 1 serum is diluted with broth and used as a culture medium for type 1 pneumococcus, the protective action of the serum is neutralized or destroyed.

[17] J. Infect. Dis., 1926, 38, p. 131.

THE EFFECT OF ZINC IN EXPERIMENTAL SYPHILIS

JOHN E. WALKER

From the Department of Clinical Pathology and Preventive Medicine, Army Medical School, Washington, D. C.

Walbum and Mörch [1] reported that salts of various metals increase the production of antitoxin and agglutinin when injected intravenously in animals previously immunized with a specific antigen. Manganous chloride gave somewhat better results than other salts. The usual dose was 1 cc. of a 0.001 M solution per kilo. The authors state that they use this method as an aid in the routine production of diphtheria antitoxin. Perlzweig [2] obtained similar results with magnesium chloride in the production of antipneumococcus serum. Mackie,[3] in experiments bearing upon the nonspecific stimulation of a natural antibody (anti-sheep amboceptor in the rabbit) found increased production after the injection of manganous chloride and of other substances, but the results were not constant. He believes that the effects of metallic salts are to be correlated with the action of heterogenetic antigens (Forssman phenomenon), and he brings all these observations together as a sort of logical basis for the use of nonspecific therapy in humans.

Several syphilitic rabbits were available, and the results of Walbum and Mörch suggested the injection of some of the salts used by them, in order to ascertain whether the possible increased antibody production would influence the course of this experimental disease.

Three rabbits with advanced unilateral scrotal chancres (Nichols strain) were given intravenous injections with 1 cc. of 0.001 M solutions of manganese, nickel, and zinc chloride per kilo, respectively. There was no effect on the lesions or spirochetes. The injections were then repeated every fourth day, the quantities of the salts being tripled each time. The fifth injection of manganese and the sixth of nickel killed the animals without having previously caused any change in the spirochetes or lesions. The experiments with zinc are given in detail.

Rabbit 1.—1st day: Ulcerated chancre on right scrotum, indurated, 2 cm. in diameter, showing numerous spirochetes. 1 cc. of 0.001 M solution of zinc chloride per kilo intravenously.

[1] Ann. de l'Inst. Pasteur, 1923, 37, p. 396.
[2] Bull. Johns Hopkins Hospital, 1925, 36, p. 322.
[3] J. Hyg., 1925, 24, p. 176.

2nd and 3rd days: No change in lesions. Spirochetes numerous.

4th day: Lesions perceptibly larger. 1 cc. of 0.003 M solution zinc chloride per kilo intravenously.

5th to 7th day: No change. Spirochetes numerous.

8th day: 1 cc. of 0.009 M solution zinc chloride per kilo.

9th day: Original lesion has increased enormously in size. Now about 3 cm. in diameter, red and edematous. Small edematous area 0.5 cm. in diameter on skin of left scrotum, positive for spirochetes.

10th day: Original lesion now 3.5 cm. in diameter. 2 small nodules palpable in testicular substance on left. Aspiration shows them to be positive for spirochetes.

11th day: 1 cc. of 0.027 M solution zinc chloride per kilo.

14th day: No definite change in lesions. Spirochetes numerous on both sides. 1 cc. of 0.081 M (0.011 Gm.) zinc chloride per kilo.

15th day: Lesions definitely look less inflamed. No spirochetes found after prolonged search.

Spirochetes were not found on seven subsequent daily examinations. Lesions continued to regress, and ulceration was entirely healed by the 30th day. This animal relapsed. On the 67th day a small nodule was palpable in the right testicle. This was positive for spirochetes.

The marked increase in the severity of the lesion with extension to the opposite side after small doses, together with the sudden disappearance of the organisms and healing of the lesions after larger doses, seemed to justify further experiments on the action of zinc. Two attempts to repeat the "Herzheimer" reaction in other animals by giving small doses as on the 8th day above were without success. The original experiment of beginning with very small doses and repeating with increasing dosage was, however, not tried again.

Rabbits 2 and 3.—Bilateral chancres, 0.5 to 2 cm. in diameter. Single injections of 0.011 Gm. zinc chloride per kilo. Permanent disappearance of spirochetes after 24 hours and 48 hours, respectively, and gradual healing of lesions. No relapse during an observational period of 100 days.

Zinc chloride, 0.033 Gm. per kilo (three times the apparent curative dose in rabbits 2 and 3), produced instantaneous death in a normal rabbit. The doses in the foregoing experiments were measured by diluting a stock M/10 solution of zinc chloride. The M/1 solution was unfortunately prepared by weighing out a somewhat moist laboratory specimen of the salt. The figures given are therefore greater than the actual amounts of zinc chloride injected, though their relative proportion is correct. Further experiments were carried out with zinc acetate, partly on account of this deliquescent character of zinc chloride, but also because it was hoped that an organic salt might show less toxicity for the animal.

Rabbit 4.—Bilateral scrotal chancres. 0.025 Gm. zinc acetate per kilo. Spirochetes disappeared in 48 hours, and remained absent on subsequent daily examinations. Lesions began to regress rapidly on the third day. This animal was found dead on the 10th day. Necropsy showed nephritis.

Rabbit 5.—Bilateral scrotal chancres, 1.5 cm. in diameter. 0.002 Gm. zinc acetate per kilo intravenously. Permanent disappearance of spirochetes after 48 hours, with rapid healing of lesions. No relapse during an observational period of 100 days. This animal showed obvious loss of weight for several weeks after the injection, but recovered completely, and was fat and healthy at the end of the experiment.

Rabbit 6.—Bilateral scrotal chancres, 1 cm. in diameter, ulcerated and indurated; 0.020 Gm. zinc acetate intravenously; no visible effect on the organisms or lesions.

The fatal dose of zinc acetate is taken as 0.025 Gm. per kilo (rabbit 4) though Wherry [4] found that a rabbit tolerated 0.030 Gm. I injected a normal rabbit with 0.030 Gm. per kilo, and death followed in 7 days. 0.044 Gm. per kilo produced instantaneous death.

A few experiments were done with subcutaneous injections of metallic zinc (zinc dust, Baker's "analysed") suspended in olive oil. This substance was not nearly so active as had been anticipated from the effects of the soluble salts. 0.300 Gm. per kilo caused the disappearance of spirochetes after 7 days, and healing of lesions. This animal died 3 months after the injection, after having developed marked emaciation and extreme alopecia of irregular distribution. Necropsy showed large deposits of zinc at the site of injection, from which it was evident that only a small portion of the zinc had been absorbed. Injection of 0.200 Gm. per kilo into another animal had no effect on the organisms or lesions. Subcutaneous emphysema at the site of injection was a constant feature in animals receiving large doses of zinc dust. The nature of the gas was not determined.

In a single experiment zinc oxide, 0.035 Gm. per kilo, given subcutaneously had no effect. Another animal received 0.032 Gm. of zinc sulphanilate (kindly furnished by Burroughs, Welcome and Co.) without observable effect. This animal did not die, but for a period of 4 or 5 days showed frequent clonic convulsions, ending with prolonged squealing. Possibly these attacks correspond to "zinc ague" seen in industrial zinc poisoning.

The rapid disappearance of spirochetes in rabbits 1, 2, 3, 4, and 5, with permanent healing of the lesions in rabbits 2, 3 and 4, indicates fairly clearly an antisyphilitic action on the part of zinc salts. Lymph gland transfers to other animals as an additional criterion of cure were not made.

Since rabbit 5 was apparently cured by 0.022 Gm. of zinc acetate per kilo, while rabbit 4 was killed by 0.025 Gm., the therapeutic index

[4] Cited by McCord et al., Arch. Int. Med., 1926, 37, p. 641.

(tolerated dose divided by the curative dose: T/C)* for zinc acetate is in the neighborhood of 1. It is probable that the same figure holds for zinc chloride, though I did not accurately ascertain its fatal dose.

Zinc salts have been previously tested in a very limited manner in experimental syphilis without observable effect by Kolle and Ritz [5] and by Klauder.[6] I do not feel that their negative results seriously conflict with the experiments here reported, for the detection of any antisyphilitic action by a substance, the therapeutic dose of which is approximately the same as the fatal dose, is largely a matter of accident.

There is to my mind no indication for the trial of zinc in human therapy. The effect of zinc, however, is believed to be of interest theoretically, chiefly in connection with previous work with mercury in experimental syphilis. It would seem that in the experimental animal zinc is more efficacious than mercury, for Nichols [7] found that the maximum tolerated dose of the bichloride was 0.001 Gm. per kilo, while it required three times this amount to produce disappearance of the organisms before the eventual death of the animal, giving a therapeutic index (T/C) of $\frac{1}{3}$. Mercury salicylate was somewhat better, having a therapeutic index of $\frac{2}{3}$. Flumerin (disodium salt of hydroxymercuriflourescein) [8] seems to have a therapeutic index nearer 1.

There are four drugs of major importance in the therapy of syphilis. Of these, mercury and potassium iodide rest on a sound empirical basis of observation over a long period of years rather than on favorable indications from animal experiments. The remaining two drugs, salvarsan (including other arsphenamines) and bismuth, were introduced into human therapy after highly favorable results in animals. These results in animals were soon confirmed by use in humans. Efforts in human therapy at present, it seems, should be directed toward finding the proper place for bismuth in the scheme of treatment, rather than to the introduction of other drugs with less promise, as indicated by lower therapeutic indexes. It may be of interest, however, to briefly review the literature of other substances known to have a feeble antisyphilitic action.

* There seems to be no general uniformity as to the manner of expressing the therapeutic index, since some authors use the reciprocal of the formula above. The formula given here seems much superior, since fractions are to a large extent avoided, and the desirable feature of relative low toxicity increases (rather than decreases) with numerical increase in the therapeutic index.

[5] Deutsch. med. Wchnschr., 1919, 45, p. 481.
[6] Arch. Dermatol. & Syph., 1924, 9, p. 219.
[7] J. Exper. Med., 1911, 14, p. 196.
[8] Hill and Young: J. Am. M. A., 1923, 80, p. 1365.

Vanadium.—The work of Proescher, Seil, and Stillians[9] seems to indicate that sodium hexavanadate has a therapeutic index of approximately 1. Fournier, Levaditi, and Schwartz[10] found that potassium tartro-vanadate had a therapeutic index of 2. Effective results are reported by both groups of workers in human therapy.

Gold.—From Truffi's[11] experiments it may be roughly inferred that gold chloride has a therapeutic index of about 1, a result also indicated by the experiments of Kolle and Ritz,[5] and of Klauder.[6] Kolle and Ritz found an index of approximately 1 for colloidal gold. Bruck and Glueck[12] had previously reported beneficial results from the use of potassium and gold cyanide in human therapy. As pointed out by Truffi, the use of gold in syphilis has a long historic background. Levaditi[13] reported a therapeutic index (T/C) of 2 for gold and sodium thiosulphate—sanocrysin. Disappearance of spirochetes and improvement of lesions were observed in humans.

Silver.—Kolle and Ritz[5] found the following therapeutic indexes: silver citrate, $\frac{3}{4}$; silver nitrate, $\frac{3}{2}$; protargol, 1; kollargol, 3. This work forms part of the theoretic basis for the use of silver salvarsan.

Platinum.—Levaditi[13] reports a definite antisyphilitic action by platinum and and sodium thiosulphate. This action, however, is less than that of gold and sodium thiosulphate.

Thorium.—Klauder's[6] results apparently indicate that thorium chloride injected intramuscularly has about the same effect as gold chloride.

Antimony.—Klauder reports that antimony tartrate injected intramuscularly has a therapeutic index of 1. It is less effective when given intravenously.

Cadmium.—The element is added merely because it seems that it is being used in therapy. Kochman and Grouven[14] reported an antisyphilitic action from cadmium subsalicylate in humans, but only when combined with salvarsan. Lucke[15] obtained similar results. The claim that a drug exerts antisyphilitic action when combined with salvarsan is very difficult to substantiate, and likewise difficult to disprove. Levaditi, Nicolau, and Navarro-Martin[16] were unable to find any antisyphilitic action of cadmium in experimental animals.

The distribution of these elements in the periodic table seems to render premature any effort to correlate chemical properties with antisyphilitic action.

Levaditi,[13] on analyzing the tissues of syphilitic animals previously treated with bismuth, found such infinitesimal amounts of bismuth in the lesions that it was difficult to conceive of any direct action of the metal on the parasites. He believes that the destruction of the organisms is due to a catalytic effect on the part of the metal (or of other antisyphilitic drugs) which accelerates the normal lytic processes by body

[9] Am. J. Syphilis, 1917, 1, p. 347.
[10] Compt. rend. Soc. de biol., 1922, 87, p. 231.
[11] Pathologica, 1913, 5, p. 397.
[12] München. med. Wchnschr., 1913, 60, p. 57.
[13] Lancet, 1926, 1, p. 215.
[14] Deutsch. med. Wchnschr., 1925, 51, p. 427.
[15] Ibid., p. 1873.
[16] Compt. rend. Soc. de biol., 1925, 93, p. 233.

tissues. This is very similar to the conception of Walbum and Mörch [1] regarding the action of metallic salts in increasing the production of immune bodies. That Walbum and Mörch did not find this action confined to a small group of metals is especially interesting in connection with the fact that the list of antisyphilitic drugs is being gradually extended. It is entirely possible, in my opinion, that there is some connection between these observations of Walbum and Mörch and the effect of curative agents in syphilis. All of these effects may be due to a stimulation of normal immune processes, a view already expressed by Pearce [17] concerning potassium iodide. The views of Mackie [3] concerning nonspecific therapy recur in this connection.

CONCLUSION

Zinc chloride and zinc acetate have therapeutic indexes in experimental syphilis approximating 1, a result somewhat better than previously obtained by other authors with mercury, and corresponding closely with the effect of simple compounds of vanadium, gold, and silver.

[17] Arch. Dermatol. & Syph., 1925, 12, p. 1.

THE USE OF HYDRAULIC DEVICES FOR OBTAINING MICROMANIPULATION

F. Lowell Dunn

From the Department of Clinical Investigation, University of Nebraska College of Medicine, Omaha

The present day methods for obtaining micromanipulation are in general based on the use of accurately machined metallic guides and slides together with screw feeds or geared mechanisms, or on the use of metal bars connected by springs and separated by highgrade screw feeds utilizing lever principles. The former type is illustrated by the Barber instrument [1] including its modification by Hecker,[2] and the new micromanipulator after Janse and Peterfi manufactured by Carl Zeiss.[3] The instrument developed by Chambers [4] illustrates the second type.

The hydraulic pump does not seem to have been used for obtaining finely regulated movement in the laboratory; in fact we have been unable to find any instance of its use for micromanipulation in the laboratory. The combination of a small pump forcing fluid into a large cylinder is a familiar application of hydraulics for obtaining great lifting power, and analogous devices are used in operating large gun mounts.

At first glance there are obvious advantages in the application of hydraulic devices to micromanipulation; wide latitude in fineness of adjustment, freedom from vibration because of the characteristics of fluids and the ease in introducing remote control, flexibility of design of apparatus, and cheapness of construction.

In a system consisting of two cylinders and pistons connected by tubing with all the air displaced by liquid, on pushing one piston the amount of movement of the other will depend on the inverse ratio of the areas of the two pistons. In one model tested out the smaller piston was a 10-32 machine screw and the larger was the piston of a 10 cc. luer syringe. The diameters of these were 0.170 and 0.750 inches respectively, and using the formula for the area of a circle the areas were 0.023 and 0.448 square inches. These were in the ratio of 1 to 19.5, i. e., a movement of the smaller piston of one millimeter would move the

[1] Phila. J. Sci., 1914, No. 4, B9, p. 307.
[2] J. Infect. Dis., 1917, 19, p. 306.
[3] Carl Zeiss, Leaflet Mikro 374.
[4] J. Infect. Dis., 1922, 31, p. 334.

larger piston 0.051 mm. or 51 micra. The screw had 32 threads per inch
from which it follows that one revolution will move it 0.794 mm., and
since the ratio is 1 : 19.5 the larger piston will move but 40.5 micra. It
is easy to turn a screw through an angle of 5 degrees. Such a turn will
move the larger piston but 0.000563 mm. or approximately one half
micron. This ratio of movement is the equivalent of a screw with
19.5 x 32, or 624 threads per inch. Since screws with more than 60
threads per inch are not very durable it is obvious that screw feeds have
a fairly definite limit in fineness of movement, unless a screw of large
diameter is used permitting movements through very small angles such as
are used in ruling diffraction gratings, or unless a differential screw is
used. High ratios can be constructed on lever principles but long bars
have periods of vibration which may be annoying. By varying the rela-
tive sizes of the pistons wide variations in fineness of movemnet can be
obtained. By using a 4-56 machine screw and a 2 cc. luer syringe prac-
tically the same ratio as above can be obtained with a considerable reduc-
tion in the bulk of the instrument. For coarser movements the controlling
piston need not have a screw feed.

Fluid transmission of pressure is peculiarly adapted to fine move-
ment because of the incompressibility of liquids and corresponding
absence of elastic vibration at ordinary pressures. The force of com-
pression movement is limited only by the bursting strength of the
apparatus while the suction movement is limited by the vapor pressure
of the liquid used. In the case of an oil at room temperature this is low.
The choice of a liquid is a practical point. We have considered that
greater freedom from leaks, smoother and more precise movement would
be obtained if the fluid wetted the pistons and cylinders. By the use of
metal tubing either seamless or flexible, remote control can be conveni-
ently obtained; in contrast to most micromanipulators in which the
standards holding the pipets must be touched.

The number of plunger systems necessary depends on the number
of movements desired. A mechanical stage can be operated with two
plunger systems at right angles. For movement in three dimensions three
are required, and though fine rotatory movements are not included in
micromanipulators these can be obtained with two extra plungers acting
tangentially. By the use of rocker arms the plungers can be located in
various positions. Three plungers mounted at right angles give the same
movements as the present micromanipulators.

By avoiding machined sliding ways and fine machine screws the cost
of construction is lowered and highgrade workmanship reduced to the
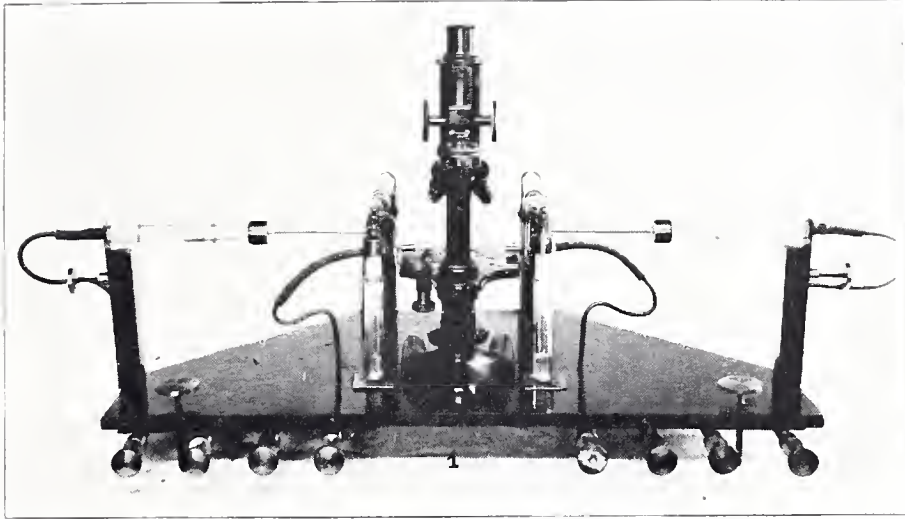
Fig. 1.—Front view large model. Controlling pistons below base. One piston serves for microinjection: 5 degree rotation displacing 0.0002 cc. fluid.
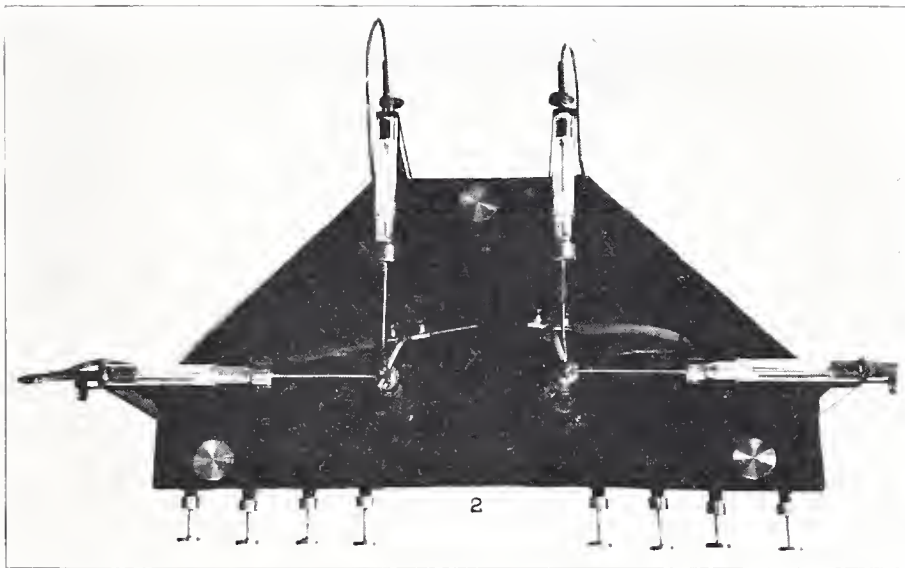
Fig. 2.—Top view large model. The large pistons are connected together by brass tubing which absorbs the strain of movement.

construction of cylinders with pistons. Good glass syringes are available on the market. A screw can be used for a controlling piston as leaking can be avoided by a packing gland.

Several instruments have been constructed on the above principles and tested out. Figures 1 and 2 illustrate a double manipulator arrangement. The base is of ½ inch steel, 24 inches long and 12 inches wide with leveling screws. The uprights are ¾ inch steel screwed to the base and held in position by steel pins. The syringes pivot in phosphor
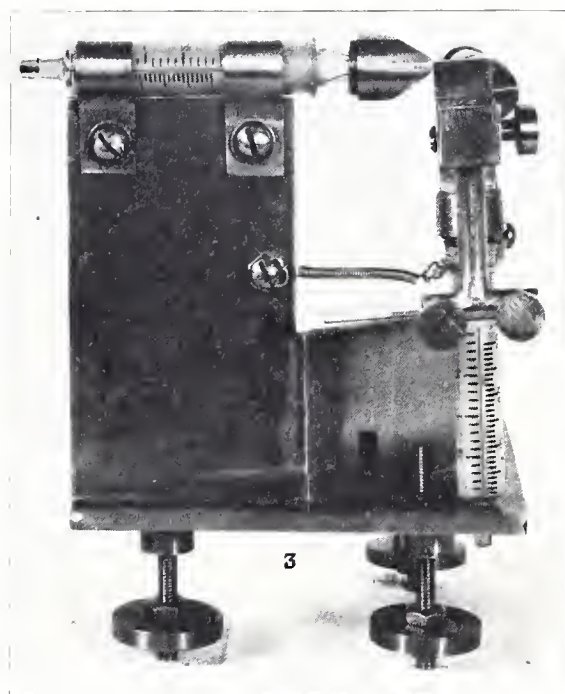
Fig. 3.—Smaller model using 2 cc. Luer syringes. Horizontal movement transmitted through edges pressing against flat flaces on vertical piston. Controlling pistons are not shown.

bronze strips and as will be seen in the figure the screws in the uprights permit coarse adjustment. The 10 cc. luer syringes are mounted at right angles and the horizontal syringes are connected by small brass tubing to the vertical syringe to which a pipet holder is attached. The screw plungers are 10-32 machine screws mounted under the base and connected by seamless copper tubing to the syringes through a short piece of pressure tubing and a ground metal to glass joint. Each screw piston has a packing gland. One screw piston connects to the pipet and serves for microinjection or suction. One revolution of this 10-32 screw by calculation displaces 0.0116 cc. and 5 degrees rotation displaces

0.0002 cc. The microscope is clamped between the vertical syringes and the systems are filled with paraffin oil.

A much more compact and more practical design is illustrated in figure 3. The base is $\frac{3}{16}$ inch brass and measures but 4 inches on the two sides. Here 2 cc. luer syringes are used and the two horizontal ones are clamped to the uprights tightly. Knife edges are attached to the horizontal pistons and press against the flat faces of a square rod attached to the vertical syringe. The vertical syringe pivots on the base and is kept in contact with the horizontal pistons by springs. The control is remote and connected to the manipulator by $\frac{1}{8}$ inch flexible metallic tubing. The controlling mechanism consists of six pistons and cylinders fitted with screw feeds and knurled wheels for adjustment. Three pistons are 0.500 inch diameter and are used for making coarse adjustments throughout a $\frac{3}{4}$ inch cube which is the range of this particular instrument. The three pistons for fine movement are 0.125 inch diameter. These six pistons are mounted on a suitable base which may be screwed or clamped to a rigid mount. The length of the connecting tubes are determined by the setup desired.

In practice the movement is conspicuously free from vibration at a magnification of 1,100 diameters and with a hydraulic ratio of 1 : 19.5 manipulation is easy. No jerkiness in movement was noted and lag was very slight. Air bubbles in the oil cause lag but practically they are easily avoided. Leaks are not troublesome because of the viscosity of the paraffin oil. The large instrument after standing two months showed no appreciable lag.

In order to test out critically the quality of the movement a steel piston of 0.875 inches diameter was carefully fitted to a brass cylinder, a plane mirror was attached to the piston, and this made the movable arm of a Michelson interferometer.[5] A small piston of 0.125 inch diameter was similarly fitted into a brass cylinder. This piston was made long and a part $\frac{1}{2}$ inch diameter was threaded with 40 threads per inch. A three inch wheel graduated into 100 parts was driven on and locked to this piston. The large and small cylinders were then connected by $\frac{1}{8}$ inch inside diameter seamless copper tubing by soldering. The system was then filled with paraffin oil. A movement of one division on the graduated wheel moved the small piston 0.00025 inch. The ratio here was about 1 : 50 and a movement of one division on the smaller piston would move the larger but 0.000005 inch or one two hundred thousandth of an

[5] Phil. Mag., 1882, 13, (5th series), p. 236.

inch. The green line of mercury (5460.74 $\mu\mu$) was used for a light source and the interferometer was adjusted for parallel fringes. The movements under these critical conditions showed no visible vibration or drift, and were prompt and decisive. Vibration amounting to a millionth of an inch would be conspicuous under these conditions if present. The backlash in the screw amounted to less than a hundredth of a turn, i. e., less than a two hundred thousandth of an inch. Counting fringes showed that the error of measurement was well within the probable error of the screw which was not carefully made or polished. Thus the interferometer which is probably the most precise device for measuring fine movement known, shows that the use of a hydraulic feed gives movement conspicuously free from vibration and lag and with a precision that appears to be determined by the quality of the screw. By using hydraulic ratios it is reasonable to believe that certain periodic errors inherent in screw feeds may be minimized as larger angular turns of the screw may be used. For precision work of course, temperature control is necessary as in all interferometer work.

SUMMARY

Finely regulated movement can be obtained by using a system of two cylinders and pistons connected together and filled with a liquid, the ratio of movement varying inversely as the areas of the controlling piston and the driven piston.

Hydraulic feed permits large variations in fineness of movement, design of apparatus, and simple design of remote control with consequent great adaptability.

When tested with a Michelson interferometer, vibration was scarcely seen and less than a millionth of an inch in amplitude. Drift was not noted and lag was barely perceptible, and the movement appeared as precise as the screw feed on the controlling piston.

By avoiding accurately machined guides and highgrade screws as in the mechanical models and utilizing hydraulic devices fine movement can be obtained more cheaply and accurately.

A BACTERIOLOGIC STUDY OF PULMONARY EMBOLISM

TWO PLATES

Edward C. Rosenow

From the Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minnesota

The etiology of the formation of intravascular clots in cases of postoperative thrombosis and embolism is still a deep mystery. Infection, while generally conceded to be the chief cause of thrombosis and embolism in sepsis, is believed not to play an important part in the formation of the large postoperative thrombi which often cause death from pulmonary embolism, for in these cases the evidences of infection, such as fever and pain from phlebitis are slight or wholly absent, and cultures from the blood and other tissues, as usually made at necropsy, are sterile. The accident is about as prone to occur in clean cases, such as following herniotomy, as in infective cases, such as following operation for acute appendicitis. Stasis from enforced rest in bed, especially emphasized by Aschoff, lowered blood pressure, vascular injury, increased coagulability and fibrin content of the blood incident to the operation, hypertrophy of the heart, and arteriosclerosis incident to advanced age are generally considered as important etiologic factors. The occurrence of cases of pulmonary embolism in groups, especially when certain respiratory infections are prevalent, speaks for a microbic etiology of the disease. If the disease is infective in origin the microorganism must have mild and peculiar invasive power, differing greatly in its action from organism associated with thrombosis secondary to septic processes.[1]

It is a noteworthy fact that a comprehensive bacteriologic study of such cases has not been made. The reasons for this are obvious. The number of cases in individual institutions is small; cultures from the blood made in the usual manner are mostly sterile or have not yielded a distinctive organism, and many bacteriologists even today do not appreciate the importance of making cultures from goodly amounts of tissue, and with what tenacity they hold onto the infecting organism.

[1] Aschoff, Ludwig: Lectures on pathology, 1924, p. 253.

About twelve years ago, while especially interested in work with tissue and blood cultures, I succeeded, by the use of a special technic,[2] in isolating a green-producing diplococcus of low virulence from the thrombus or blood in each of a number of cases of thrombosis (one of primary portal thrombosis [3]) and pulmonary embolism. Retrograde thrombosis of the branches of the portal vein was produced by intravenous injection in animals of the diplococcus isolated from the patient with portal thrombosis. The recent occurrence of an unprecedented number of cases of postoperative thrombosis in the Mayo Clinic impelled me again to study the question from the bacteriologic standpoint. I wish here to report the results obtained from a microscopic and cultural study of pulmonary emboli and thrombi in a series of cases in which death occurred suddenly from pulmonary embolism, or from infarction of the brain or myocardium.

Technic.—The emboli or thrombi and other tissues which were studied microscopically were fixed in a 10% solution of formalin. Blocks were embedded in paraffin and sections about 10 microns thick were stained for cellular changes with hematoxylin and eosin, and for bacteria by a modified Gram method. The latter consisted in staining deeply with gentian-violet or methyl-violet solution, fixing in the iodine-potassium-iodide solution, and decolorizing with alcohol to a pale blue instead of the end point as recommended in the Gram formula. In this way the nuclei of cells appear as pale blue, and the bacteria as dark purple, almost black, bodies. Partial decolorization has the additional advantage of making visible gram negative bacteria such as the Bacillus coli. Necropsy in nearly all cases was performed within twenty-four hours after death, the bodies in some cases being embalmed within a few hours after death. In suitable cases cultures of the blood from the heart, the embolus and thrombus, spleen and liver, were made as a routine. The blood from the heart and splenic and hepatic tissues were aspirated into sterile Pasteur pipets after the surfaces were seared. The embolus from the pulmonary artery was removed in as sterile a manner as possible, and three pieces, each about 1 cm. long, washed from three to six times by vigorous shaking in large amounts (120 cc. each) of sodium chloride solution. If the probability of contamination was marked the pieces were dipped into alcohol and the alcohol burned off at once by plunging them into a Bunsen flame.

Cultures from the thrombus as found, usually within the iliac vein, were made by the following technic. Two ligatures were placed about the vein and the portion with the thrombus in situ removed in a sterile manner. This was then dipped in alcohol and the surface sterilized by ignition of the alcohol. The wall of the vein was then carefully separated and three pieces of the thrombus, each about 1 cm. long, removed and washed repeatedly in sodium chloride solution. One of the pieces of thrombus was placed in 10% formalin, one was emulsified in sodium chloride solution with mortar and pestle, and one was cut into small blocks with sterile shears, carefully hooded under the cover of a petri dish. The blood, pipettings from the spleen and liver, the emulsions and small pieces of embolus and thrombus were inoculated into mediums affording a wide range of oxygen tension, such as blood agar plates, glucose brain broth (0.2%), soft

[2] J. Am. M. A., 1914, 63, p. 903.
[3] Lewis, D. D., and Rosenow, E. C.: Arch. Int. Med., 1909, 3, p. 232.

glucose brain agar, and into low columns of plain broth. Emulsions of thrombus and embolus were also inoculated into tall columns of 2% dextrose broth in amounts of 120 cc. each. The brain broth and agar were boiled just previous to inoculation to drive off dissolved oxygen. The surfaces of blood agar plates were streaked. Tall tubes of glucose brain broth and dextrose broth and low columns of broth were inoculated with emulsions, and shake cultures were made in tall columns of glucose brain agar (0.7%). The emulsions were inoculated in varying amounts in order to vary conditions for growth as much as possible, and pieces of antemortem clots were placed at different levels of the glucose brain agar tubes. Cultures were incubated at 35 C. Animal inoculations were made with primary cultures, or young cultures of transfers made from three to six times each day in order to prevent loss of specific infecting power.

Results of Cultures.—Cultures were made in five necropsy cases of postoperative pulmonary embolism following thrombosis of iliac or femoral veins and in one case of portal thrombosis.

Case 1.—The patient was a woman, aged 53 years, who had died suddenly from pulmonary embolism five days after abdominal hysterectomy for liomyoma of the uterus.

Case 2.—The patient was a woman, aged 68 years, whose death had occurred from pulmonary embolism eight days after amputation of the breast for ulcerating carcinoma.

Case 3.—The patient was a woman, aged 42 years, who had died suddenly from pulmonary embolism four days after vaginal hysterectomy for uterine polypus and prolapsus.

Case 4.—The patient was a woman, aged 61 years, who had died suddenly from pulmonary embolism 33 days after beefbone pegging for ununited fracture of the femur, a clean operation.

Case 5.—The patient was a man, aged 59 years, who had died two days after herniotomy for bilateral inguinal hernia.

Case 6.—The patient was a man, aged 49 years, who had died from portal thrombosis and peritonitis seven days after partial gastrectomy for carcinoma of the stomach.

Necropsy in all six cases was performed within twelve hours after death. In only two was there evidence of sepsis. The patient with ulcerating carcinoma had developed cellulitis in the operative field due to hemolytic streptococci, and the patient with gastric carcinoma had developed peritonitis in addition to portal thrombosis. In the others clinical and postmortem evidence of infection was slight or absent, and the development of the thrombus was not suspected before the vascular accident occurred.

A diplostreptococcus, similar to the one obtained twelve years ago in similar cases in Chicago, was isolated from the embolus or thrombus in each of the five cases of pulmonary embolism and from the thrombus in the case of portal thrombosis (fig. 1). The number of colonies of the diplostreptococcus was not large, never more than 500 for each gram of thrombus, and in the primary culture, growth occurred first in the deeper layers of the glucose-brain-agar shake cultures of emulsions and usually began in the bottom of glucose brain broth. On longer incubation, if the total number of colonies was relatively large, colonies appeared at higher levels and the growth in glucose brain broth extended to the top. Colonies could be seen growing from the depths of the pieces planted into the soft glucose brain agar. Primary cultures on blood agar and in low columns of broth never yielded the diplostreptococcus but subcultures from colonies in the glucose brain agar or from the glucose brain broth grew readily on these mediums. A staphylococcus and the Bacillus coli were isolated from emulsions of embolus and thrombus in two cases.

The blood from the heart, liver, spleen and kidneys was sterile in four cases. The diplostreptococcus was obtained from the blood in one instance only. In the case in which death occurred suddenly from pulmonary embolism following amputation of the breast for ulcerating carcinoma and in which marked cellulitis with septic fever occurred, a hemolytic streptococcus was isolated from the infected tissues and blood. in pure culture, and from the pulmonary embolus and iliac thrombus together with the diplococcus. In the case of portal thrombosis associated with peritonitis following gastrectomy for carcinoma of the stomach, Bacillus coli, Bacillus welchii and the diplostreptococcus were isolated from the thrombus. Cultures from the blood yielded Bacillus coli only. The diplostreptococcus isolated in the different cases were much alike. It is a gram-positive diplostreptococcus resembling the pneumococcus closely in size, shape and grouping. It forms short chains in broth, is free from a capsule, and individual cocci are not so lancet-shaped as the pneumococcus (fig. 1). It produces small dry nonadherent colonies on horse-blood-agar plates. These are usually surrounded by a green or brownish zone of partial hemolysis on isolation. Later the growth may be indifferent to blood agar. Two of the strains fermented inulin and salicin; one of these also fermented mannite; the rest did not ferment these carbohydrates. All fermented dextrose, lactose and raffinose.

Animal Experiments.—Attempts to produce, thrombosis were made in mice, guinea-pigs, rabbits, and dogs, with 4 of the 6 strains of the diplococcus. Single and repeated inoculations in varying amounts were made intravenously, intraperitoneally, intratracheally, intra-ocularly, subcutaneously and intracerebrally. The clotting time of the blood obtained from the ear was determined before and after injection. As it was thought that chronic foci of infection, present in pronounced form in most cases studied, might harbor this organism and thus increase the chances of the development of thrombosis, injections were made into the posterior chamber of the eye of a number of rabbits, and the teeth of several dogs were infected with the diplococcus from such foci. Various procedures believed to favor the antemortem formation of clots were used coincidentally with the bacterial inoculations. Prolonged anesthesia, especially with urethane, was induced. Operations, such as hysterectomy, gastroenterostomy, and anastomosis of blood vessels, were performed on rabbits and dogs. Venous stasis was produced by placing rubber bands about the leg on the side of the injection. Since the forma-

tion of clots in human beings usually begins in radicles of the iliac or portal veins, hemorrhoidal vessels were injured in the animals.

None of 13 mice given intraperitoneal injections with from 1 to 1.5 cc. of the broth culture developed thrombosis, and only 3 died from peritonitis. The rest remained well.

Four of 18 guinea-pigs given from 1 to 5 cc. of the glucose-brain-broth cultures developed lesions which appeared to be secondary to thrombosis of vessels. Only 4 died from infection due to the diplostreptococcus injected. In one, marked hemorrhagic infiltration over a sharply circumscribed area along the greater curvature of the stomach was found after five daily intravenous injections of 1 cc. In one, hemorrhage with beginning necrosis was found. In one that died six days after the last of four daily injections of 1 cc. of a culture in the fourth to the sixteenth subculture the transverse colon was hemorrhagic for a distance of 5 cm., the vessels leading to this area were filled with firmly clotted blood and sections revealed the diplococcus in the hemorrhagic lesions. In one there was thrombosis of the superior vena cava which appeared to be due to marked infiltration of the posterior mediastinum secondary to bronchopneumonia and pericarditis eleven days after intratrachial injection of 2 cc. of the primary glucose-brain-broth culture of the diplococcus isolated from the pulmonary embolus in case 5. The diplococcus was isolated in pure culture from the blood and thrombus.

Of the 26 rabbits given injections, nine died from the effects of the diplococcus, and in nearly all the right auricle and ventricle were markedly dilated owing to firmly clotted blood. Some of these blood clots were laminated, mottled in color, dull and granular on the surface, firm in consistency and adherent, including the clots found in animals that died just previous to examination. The fact that examination was carried out so soon after death seemed to indicate that the clots were formed before death, although sometimes it was impossible to be certain as to this point. In four, lesions were found that clearly appeared to be due to thrombosis or embolism. In one rabbit a large adherent antemortem clot was found in the right ventricle and extending into the pulmonary artery for a distance of 4 cm. The sections of the thrombus showed circumscribed areas of leukocytic infiltration, and Weigert's stain showed numerous deep blue threads and bands of fibrin. Seven days previously this rabbit had been given an injection in the right eye with 0.2 cc. of the glucose-brain-broth culture isolated from the iliac thrombus in case 4, and the animal was kept anesthetized with urethane for three days. Besides the huge thrombus in the heart, it had what appeared to be an infarction, 10 cm. long, in the descending colon. Cultures taken from the blood after death were sterile; those from the thrombus revealed a pure culture of the diplostreptococcus, and sections from the thrombus and lesion in the bowel stained by Gram's method revealed the same organism in, or adjacent to, the lesions and not elsewhere. One rabbit that received several intravenous injections developed thrombotic outgrowths from the cordae tendonae and papillary muscles in the left ventricle and paralysis of the hind extremities, probably due to infarction. One had what appeared to be an infarction of the rectum, one an infarction with perforation of the transverse colon, and one a gangrenous intestinal loop secondary to intussusception. Sections of lesions in the intestines and of the antemortem clots, including thrombi in small vessels, and in infarcted areas, revealed the diplococcus. The joints, heart valves, stomach, gallbladder, and liver were almost wholly without lesions, in spite of repeated intravenous injections.

In one rabbit large thrombi, mottled grayish red and granular on the surface, were found in the dilated right ventricle, left auricle and inferior vena cava. The thrombus in the right ventricle was adherent to the septal wall and extended into the pulmonary artery for a distance of 2.5 cm. The one in the left auricle was

adherent to the endothelial lining near the apex. There were no lesions of the viscera and cultures of the blood from the heart proved sterile. Sections of the thrombi revealed areas of leukocytic infiltration of varying degree, marked fibrin formation and scattered gram-positive diplococci singly and in short chains (fig. 2). Sections through the septal wall at the point of attachment of the thrombus in the right ventricle revealed thrombosis of small vessels and a large vein (fig. 3) adjacent to a large branch of the coronary artery and in which a large number of gram-positive diplococci were found. A small area of the intima was found infiltrated by leukocytes and large numbers of diplococci. This animal had received two subcutaneous injections six and five days before death, of the streptococcus isolated from the iliac thrombus in case 3. The culture injected was obtained from a single colony in the deeper layer of the soft glucose brain agar that had been inoculated the day before with an emulsion of the iliac thrombus from the patient and had been rapidly subcultured three times in glucose brain broth of which 1 and 2 cc. were injected.

Seven dogs were given injections with from 2 to 20 cc. of glucose-brain-broth cultures of three of the strains. Four were operated on just previous to the first injection and three were not. Three developed thrombosis of veins and two, thrombosis and embolism of the pulmonary artery. In one dog formation of a thrombus began at the point of end-to-end anastomosis of the jugular vein. The thrombus was firm, mottled, grayish red and granular, and extended proximally into the superior vena cava. Several fair sized antemortem clots were expressed from the pumonary artery; the largest of these was 0.8 cm. in diameter and 3 cm. in length. The animal had received two intravenous injections of 5 cc. of the diplostreptococcus isolated in case 5, one two days before, the other just before death. There were localized areas of edema of the lung resembling infarcts from the plugging of branches of the pulmonary artery. Cultures from the blood and thrombus yielded a pure growth of the diplococcus and sections stained by Weigert's method revealed marked deposition of fibrin.

In one dog thrombosis began around the esophagus on the posterior aspect of the mediastinum, extended into the superior vena cava and ended abruptly in a cup-shaped end at the opening into the right auricle (fig. 4a). Similar but loosely adherent thrombi were found in the inferior vena cava and iliac vein (fig. 4b) and the portal vein. A long antemortem clot was expressed from the pulmonary artery together with smaller ones (fig. 4c). The right middle lobe of the lung showed areas of bronchopneumonia, and in the right lower lobe were found several wedge-shaped hemorrhagic areas resembling recent infarcts. The animal was injected intravenously daily for six days with 5 cc. of the strain in case 5 in from the fourth to the sixteenth subculture. It died two days after the sixth injection. Cultures from the blood yielded the Bacillus coli, from the thrombus in the superior vena cava Bacillus coli and the diplococcus, and from the liver and infarcted areas the diplococcus only.

In one dog large antemortem clots were found in the radicles of the iliac vein, associated with small areas of hemorrhagic infarction in lungs and broncho-pneumonia. Gastroenterostomy had been performed five days previously, and four intravenous injections of the strain in case 1 in the fourth to the sixteenth subculture were given. The clotting time of the blood dropped from four minutes the day after the first injection to one and a half minutes the day before death. Cultures from the blood and thrombus yielded the diplostreptococcus injected. Sections revealed areas of leukocytic infiltration in the thrombus. areas of moderately diffuse infiltration in the intima of the wall of the vein (fig. 5), and marked fibrin formation (fig. 6) and diplococci within areas showing fibrin deposit (fig. 7).

None of the dogs in which the teeth were infected with the diplostreptococcus developed thrombosis. In no instance did thrombosis develop in the femoral or other veins on the side where venous stasis was induced by means of rubber bands, even in animals in which the constriction was continued for several days and in which moderate edema of the leg developed.

Nine animals (3 guinea-pigs, 4 rabbits, and 2 dogs) were given injections of freshly isolated heterologous strains from ten days to two weeks after the last of the first series of injections. All remained well and free from thrombosis and embolism. In some instances filtrates or cultures of freshly isolated strains hastened clotting of blood in vitro.

The thrombi produced experimentally were similar in gross and microscopic appearance to those found in the cases of human beings. They were firm in consistency, granular and opaque on the surface, mottled with alternating grayish and red areas, sometimes laminated, and loosely adherent to the intima of vessels. They grew to large size as early as two days, and as late as twelve days, after the first injection of the organism. Areas of softening were not found. The presence or absence of fibrin in the thrombi was determined by means of Weigert's special stain. Fibrin deposits and areas of leukocytic infiltration were usually marked (figs. 2, 3, 5, and 6), similar to those in thrombi in the cases of human beings. The wall of the vein at the point of attachment showed more cellular infiltration than it did in patients, although this was always slight in the animals (fig. 5). Diplococci, singly, in small clumps, and in short chains, were found (figs. 2 and 7) in the experimentally produced thrombi in each of the three species of animals. This was true in some instances even when cultures of the blood from the heart were sterile. They occurred chiefly in, or adjacent to, areas of fibrin deposit, less commonly in areas of leukocytic infiltration in thrombi and within endothelial cells in the intima of vessels or wall of the heart at the point of attachment of the thrombus.

On the basis of these findings it was thought worth while to study, microscopically, pulmonary emboli and thrombi that had been preserved in a 10% solution of formalin and which were derived from other similar necropsy cases during 1925. In none of the 25 cases studied had septic fever developed, and in most of them death had occurred from embolism without surgical complications.

In nineteen of the cases death resulted from postoperative pulmonary embolism in from 5 to 26 days after operation. In eighteen the operation was an abdominal one, such as gastroenterostomy, cholecystectomy, hysterectomy or splenectomy. In one the operation was the removal of a cervical rib. The ages of the patients ranged from 32 to 72 years; twelve were less than 50 years of age. No operation was performed in six cases, in three of which death from pulmonary embolism

was sudden. One of the latter was a case of carcinomatosis, the patient a man aged forty; one a case of malignant hypertension associated with cholecystitis and ulcer of the stomach, the patient a woman aged thirty, and one a case of tumor of the pituitary, the patient a man aged forty years. One patient died from infarction of the brain, lungs, spleen and kidneys, associated with antemortem thrombi in the right and left auricular appendices, one from infarction of the left ventricle, and one from myocarditis associated with thrombi in the right and left auricles in a case of hyperthyroidism.

The gross and microscopic pictures of the thrombi were typical. They showed lamination, circumscribed areas of leukocytic infiltration of varying intensity including veins and heart valves at the point of attachment of the thrombus (fig. 8) and marked deposits of fibrin (fig. 9), but nowhere was there softening due to the formation of pus.

A gram-positive diplostreptococcus morphologically identical to the one isolated and demonstrated microscopically in sections in the six cases cultured was found in the pulmonary embolus, thrombus, or infarcted area in all but two of the 26 cases. In one of these pulmonary infarction had occurred 65 days previously, and in the other, pulmonary infarction associated with terminal pneumonia, 26 days after laparotomy. The diplococci were demonstrated in the thrombi alike in the operative and nonperative cases. They were never numerous; prolonged search was often necessary to find them. They were arranged singly, in small clumps, and occasionally in short chains (fig. 10). They were found most often where fibrin was being laid down and less so in areas of leukocytic infiltration away from the surface and in endothelium where the thrombus was attached to the wall of a vein (figs. 10 a and b). In several instances a few diplococci were found in what appeared to be vaso vasorum in the walls of the veins showing leukocytic infiltration. In the patient that died from infarction of the heart they were found in the wall of the auricle, in the infarcted area and thrombi. They were never found in normal tissue remote from lesions.

SUMMARY

By means of special methods a diplostreptococcus identical to the one previously obtained in similar cases has been isolated in each of 5 recent cases of postoperative pulmonary embolism and in one case of portal thrombosis, and has been demonstrated microscopically within the thrombus or embolus or infarcted areas in all but two of 25 additional cases. The cases are typical of the condition as it occurs elsewhere. The organism is of low general virulence. It rarely causes death in mice from peritonitis following intraperitoneal injection, nor encephalitis in

rabbits following intracerebral inoculation. It rarely causes lesions in the various tissues except those secondary to thrombosis or embolism. The blood of animals was usually sterile or contained relatively few organisms, facts in harmony with the noteworthy lack of, or mild, febrile reaction and the usual absence of the organism from the blood. The diplostreptococcus is difficult to grow in citrated or defibrinated human and horse blood in vitro. It often shortens the coagulation time of the blood of animals after repeated intravenous injection, and with pure cultures thrombosis sometimes associated with pulmonary embolism has been produced experimentally in three species of animals. Experiments have been successful with each of the four strains injected and isolated from thrombi and with one strain isolated from foci of infection at the apexes of teeth. Such results have not been obtained in numerous experiments following injection of morphologically similar organisms from cases other than pulmonary embolism.

The factors such as anesthesia, operative procedures, slowed circulation and trauma of vessels, which are considered as etiologic in human cases appeared to favor clot formation in experimental cases, but in some instances the mere injection of the organism sufficed. The experimentally produced thrombi resembled those in man in their frequent formation in large veins and their large size; in being loosely attached, leading to embolism, in gross and microscopic appearance, including the deposition of fibrin; in not leading to suppuration, in the presence of relatively small numbers of the diplococcus in pure form within the thrombus, and in the relatively slight and circumscribed areas of endophlebitis. The number of bacteria was relatively small and in some instances the production of a local focus, as in the eye, or even subcutaneous injections, sufficed to incite the formation of thrombi.

CONCLUSIONS

The conclusion seem warranted that the diplostreptococcus, isolated from postoperative emboli and thrombi, and used experimentally in different animals, is the common cause of postoperative and nonoperative massive thrombosis, leading to fatal pulmonary embolism, and perhaps of closely allied conditions such as infarction of the heart or brain. In the light of these experiments stasis and other factors generally considered as causes of this condition appear to be merely contributory. The immunity found to exist in animals ten to fourteen days after a series of injections suggests the possibility of a means of prevention through specific inoculation with a vaccine prepared from this organism.

Plate 1.

Explanation of Plate

Fig. 1.—Photomicrograph of a smear from a single colony of diplococci that grew in the depths of soft glucose brain agar from the margin of a small piece of thrombus found in the iliac vein in case 2, showing morphology characteristic of the diplococcus and the short chains. Gram stain, × 1000.

Fig. 2.—Leukocytes, red blood corpuscles, fibrin threads and gram-positive diplococci in the thrombus found in the right ventricle of a rabbit given injections, subcutaneously five and six days before, with the streptococcus from the thrombus in case 3. Weigert's fibrin stain, × 1000.

Fig. 3.—Thrombosis of one large and two small veins adjacent to a large artery in the septal wall of the heart at the point of attachment of the thrombus found in the right ventricle in the rabbit referred to in fig. 2.

Fig. 4.—Thrombi and emboli found in a dog given intravenous injections with the diplostreptococcus from the patient in case 5: a, thrombus found in the superior vena cava and its branches; b, thrombus found in the inferior vena cava; and c, embolus expressed from the pulmonary artery.

Plate 2.

Explanation of Plate

Fig. 5.—A section of thrombus in iliac vein in a dog, showing areas of localized leukocytic infiltration and mild diffuse infiltration in the intima of the wall of the vein. Hematoxylin and eosin, × 1000.

Fig. 6.—Section of iliac thrombus of fig. 5. Note the leukocytic infiltration and the irregularly distributed threads and bands of fibrin. Weigert's fibrin stain, × 200.

Fig. 7.—Diplostreptococci in experimentally produced thrombi: a, a short chain in the thrombus of a dog injected intravenously; b, diplococcus in thrombus of a rabbit injected into the eye; and c, diplococcus in the thrombus shown in fig. 4a.

Fig. 8.—Section of thrombus and auricle in a man, aged 28 years, who died from infarction of the brain, lungs, spleen and kidneys with antemortem thrombi in the auricular appendices. Note especially the small area of leukocytic infiltration in the wall of the auricle adjacent to the thrombus. Hematoxylin and eosin, × 100.

Fig. 9.—Section of pulmonary embolus from a patient with carcinoma of the thyroid who died from pulmonary embolis in twenty-one days after operation. Note especially the irregularly distributed threads and bands of fibrin. Weigert's fibrin stain, × 200.

Fig. 10.—Diplococci in the thrombus in the iliac vein of a patient who died suddenly from pulmonary embolism 15 days after gastrectomy for carcinoma of the stomach: a, diplococci along the line of attachment of the thrombus; b, diplococci in endothelial cell in the intima of the wall of the vein; c, a group of diplococci within the thrombus remote from its attachment. Gram stain, × 1000.
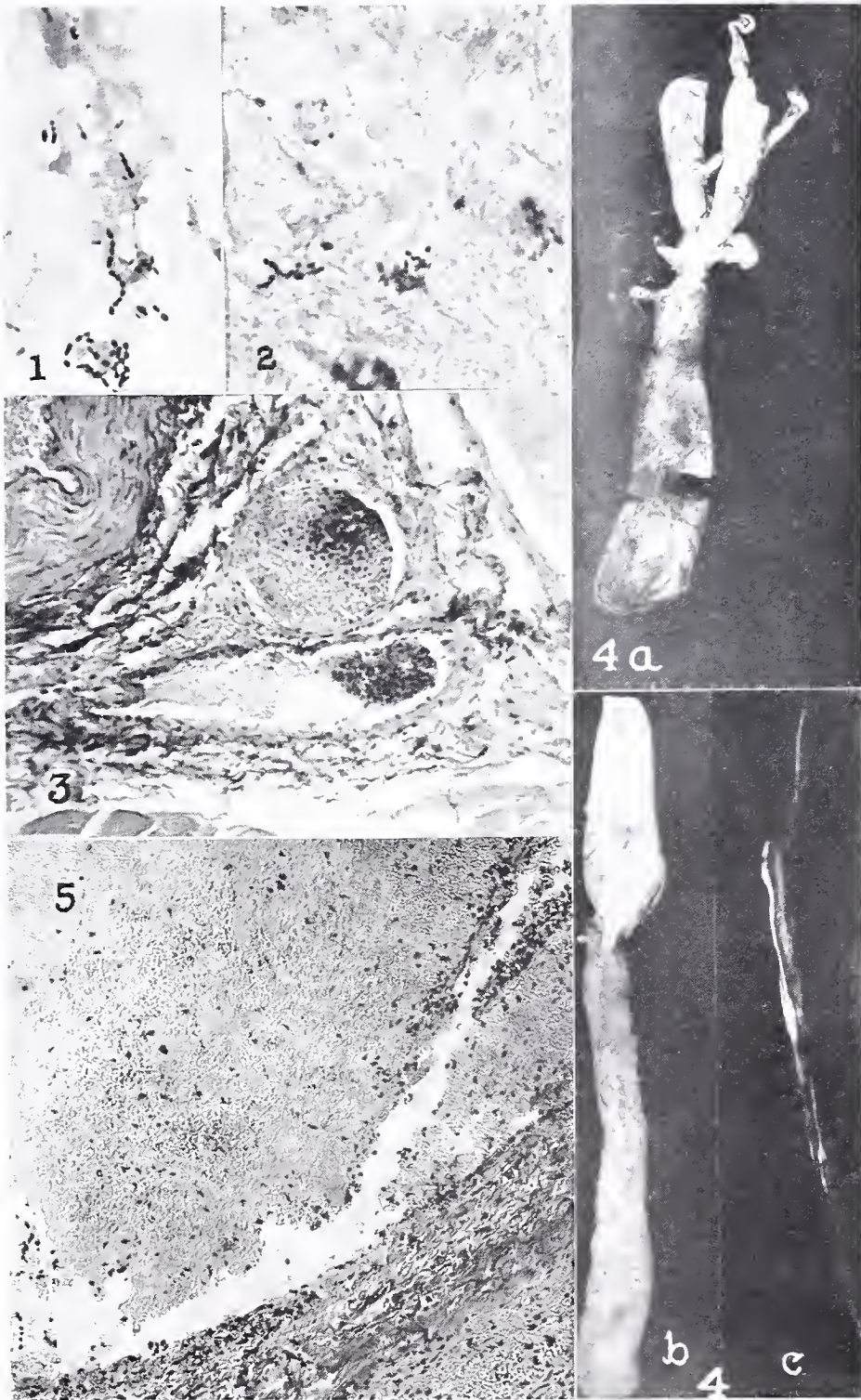
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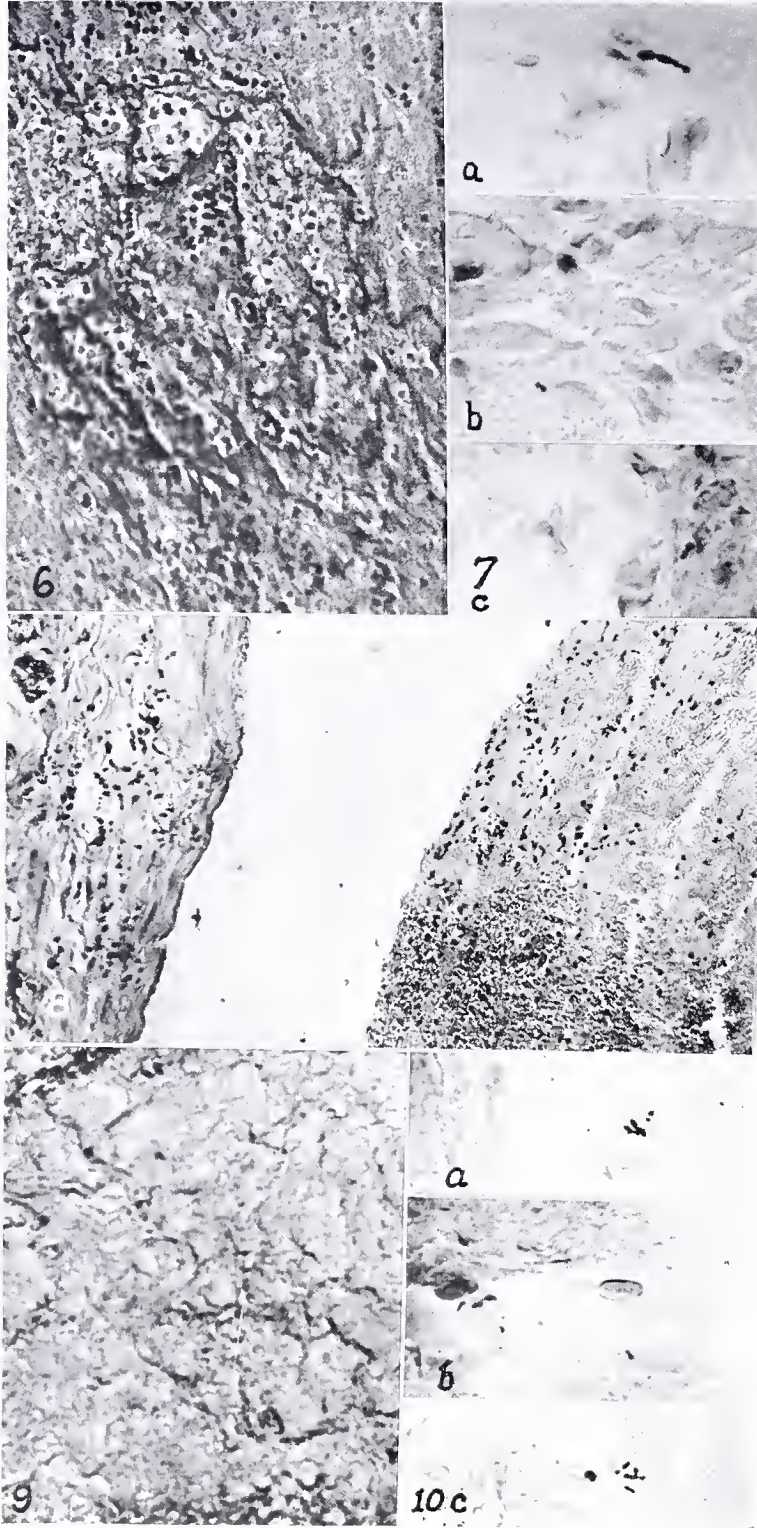
THE RESISTANCE OF DIFFERENT CONCENTRATIONS OF A BACTERIOPHAGE TO ULTRAVIOLET RAYS

RUDOLPH FISHER AND EARL B. MCKINLEY

From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York

Considerable work has demonstrated the destructive effect of ultraviolet rays upon both enzymes and living cells.

Among others Dreyer and Hanssen,[1] Kreibich,[2] Schmidt-Nielson,[3] Burge, Fischer and Neill,[4] Vedder,[5] and Allen and Ellis[6] have exposed ferments, enzymes, proenzymes and hormones to light of short wave length; while Vallet,[7] Bayne-Jones and van der Lingen,[8] Mayer and Dworski,[9] Mayer and Guttmacher,[10] Browning and Russ,[11] Cambier,[12] Burge,[13] Eidenow,[14] Weiss and Weiss[15] and several others have exposed living cultures of bacteria and other living cells to the action of ultraviolet rays. Radium too has been employed, for example by Bruynoghe,[16] who has reported the absence of lytic principle in the sixth passage of his bacteriophage filtrates when B. typhosus, the sensitive organism used, was previously exposed; and by Brutsaert[17] who exposed d'Herelle's P3 to radium emanations equivalent to 0.914 grains of radium for twenty-four hours, and found the lytic property only slightly attenuated. Insulin, according to Ellis and Newton,[18] is apparently more active after relatively short exposure to ultraviolet light, losing its ability to produce hypoglycemia only after prolonged irradiation.

The mass of this work, however, indicates that destruction takes place and is directly proportional to the amount of radiant energy applied.

[1] Compt. rend. Acad. d. Sc., 1907, 145, p. 564.
[2] Arch. f. Dermatol. u. Syph., 1912, 103, p. 529.
[3] Ztschr. f. physiol. Chem., 1908, 58, p. 233.
[4] Am. J. Physiol., 1916, 40, p. 426.
[5] Abst. J. Am. M. A., 1925, 84, p. 242.
[6] J. Am. M. A., 1925, 85, p. 94.
[7] Compt. rend. Acad. d. Sc., 1910, 40, p. 632.
[8] Bull. Johns Hopkins Hosp., 1923, 34, p. 11.
[9] Am. Rev. Tuberc., 1924, 10, p. 166.
[10] Ibid., p. 170.
[11] Arch. Radiol. & Electrotherap., 1918, 23, p. 85.
[12] Rev. Hyg., 1910, 32, p. 411.
[13] Am. J. Physiol., 1916, 40, p. 137.
[14] British J. Radiol., 1926, 31, p. 33.
[15] Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 87.
[16] Compt. rend. Soc. d. biol., 1925, 92, p. 464.
[17] Ibid., 1923, 89, p. 1173.
[18] Am. J. Physiol., 1925, 73, p. 530.

Attempts to throw light on the nature of bacteriophage by determining its behavior toward ultraviolet rays have been few. Gildenmeister [19] finds approximately equal susceptibility of lytic principle and sensitive organism (about 45 minutes). Gerretsen, Gryns, Sack and Sohngen [20] describe the death of B. radicicola after 15 minutes' exposure, though its lytic principle resists over 2 hours' irradiation. A report of Zoeller,[21] also, implies that B. shiga succumbs before its lytic principle.

This is not true of B. coli D and its bacteriophage in our experiments. As an earlier report describes, an undiluted principle, lytic for B. coli D, is completely destroyed by exposure to ultraviolet rays, while B. coli D itself survives the same irradiation.[22] Other work demonstrates progressive diminution of this lytic principle when serially diluted and further confirms and emphasizes the particulate character of bacteriophage.[23] The present report describes the effect of dilution upon the resistance of this bacteriophage to ultraviolet rays.

The principle lytic for B. coli D used in these experiments was the same described in the earlier report,[22] but increased in titre by feeding. It is regularly active in dilutions up to 1 : 100,000, and, after over night incubation with one drop of a young susceptible culture, in dilutions also of 1 : 1,000,000 and 1 : 10,000,000. Since the presence of but a single demonstrable particle gives a fully positive test after over night incubation with B. coli D, we have usually included but a single such over night incubation in the procedure described below, deeming this a satisfactory final test. In several special instances during preliminary work, however, four and five passages were made to test recovery of rayed lytic principle undemonstrable after incubation. All such tests were negative.

Method.—To determine the shortest period of exposure necessary to inactivate a given dilution, that is, the minimal lethal exposure, the following procedure was adopted. Serial dilutions in beef extract broth, from 1 : 10 to 1 : 10,[7] were made up in 100 cc. quantities. A series of quartz tubes, each containing exactly 5 cc. of the dilution to be tested, was exposed to the emanations from an Alpine sun lamp * of 4.5 amperes at a distance of one foot. The temperature of the surrounding air never exceeded 42 C. and averaged 38 C. The tubes were slanted at an angle of about 5 degrees in glass Petri dishes and were gently agitated every five minutes by sliding the dishes back and forth twenty times.

[19] Centralbl. f. Bakteriol., I, O., 1922, 89, p. 181.

[20] Centralbl. f. Bakteriol., 2, O., 1923, 60, p. 311.

[21] Compt. rend. Soc. de biol., 1923, 89, p. 860.

[22] McKinley, E. B.; Fisher, R., and Holden, M.: Proc. Soc. Exper. Biol. & Med., 1926, 28, p. 408.

[23] McKinley, E. B., and Holden, M.: J. Infect. Dis., 1926, 39, p.?????

* We are indebted to Dr. A. F. Hess for the use of his Alpine sun lamp.

The smallest difference allowed between exposures of successive tubes was two minutes. Each series was controlled by an unrayed tube and a glass tube corresponding to the terminal quartz tube, irradiated for the longest period in the series.

To determine the persistence of the lytic principle 3 tests were made from each tube. The agar slant method, described elsewhere [23] was used. Test 1 was made just after irradiation. Then each tube was inoculated with one drop of a three to six-hour culture of B. coli D and incubated 18 hours. At this point the turbidity of the unshaken tubes was noted and test 2 was made. The tubes were now heated for 30 minutes in a water-bath at 56.5 C., and finally test 3 was made. A typical series is condensed in table 1.

TABLE   1

TESTS OF 1: 10 DILUTIONS OF LYTIC PRINCIPLE EXPOSED TO RAYS FROM ALPINE SUN LAMP
FOR DIFFERENT PERIODS OF TIME

Tubes	Minutes Exposed	Turbidity at Time of Test 2	Presence (+) or Absence (0) of Lytic Principle after Exposure Tests		
			1 At Once	2 18 Hours after Inoculation	3 Inoculated Tubes Heated 30 Min. at 56.5 C.
1*	0	+++	+	+	+
2	10	+++	+ confluent plaques	+	+
3	20	+++	+ one plaque	+	+
4	30	+++	0	+	+
5	50	++	0	+	+
6	54	++	0	+++	+
7	58	+	0	+	+
8	60	+ (clearest)	0	+	+
9	62**	+++	0	0	0
10	64	+++	0	0	0
11	66	+++	0	0	0
12*	66	+++	+	+	+

* Glass tube.
** Minimal lethal exposure for dilution 1:10.

In this particular series it will be seen under test 1 that destruction begins promptly, appearing here as early as after 10 minutes, but is not complete, as shown by tests after incubation, until at least 62 minutes of irradiation. This period is therefore the minimal lethal exposure in this series. Moreover, this destruction reduces the number of lytic particles, just as does dilution,[23] so that the test shows first a wide lytic path, then a path with serrated edges, then individual but confluent plaques, and finally single, isolated plaques. It will be further observed that there is complete correspondence between tests 2 and 3, before and after heating. The fact that from 30 to 60 minutes of exposure gave negative results in test 1 but positive in test 2 is interpreted to mean simply that the drops withdrawn for the test happened to contain none of the progressively fewer particles of bacteriophage. Incidentally,

turbidity is observed to decrease toward a point just preceding complete inactivation. Contamination, of course, would mask this effect without injuring the lytic principle, as also would agitation, which stirs up the settled agglutinated clumps of resistant organisms. Finally controls not irradiated or irradiated in glass tubes, remain quite unaffected.

In three different sets of dilutions, each ranging from 1:10 to 1:10,000,000 made up with three different lots of beef extract broth A, B and C, P_H 7.6, the minimal lethal exposures were determined in this fashion with similar results. These results are shown in figure 1.
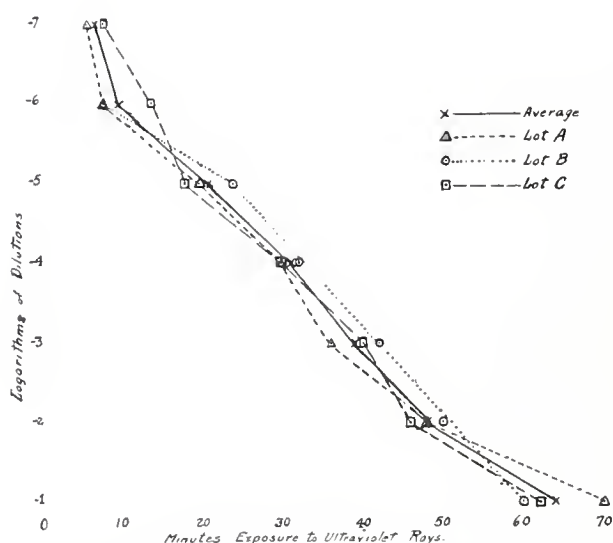
Fig. 1.—The minimal lethal exposures (abscissa) for lytic principle in dilutions 1:10 to 1:10,000,000 in three broths A, B and C plotted according to the logarithms of the dilutions (ordinate).

Several variables, difficult to exclude, contribute to error in these experiments. Differences between individual quartz tubes, none of which is without flaw and no two precisely alike; the sometimes unavoidable formation of protective vapor bubbles, outside variations in current, and the necessity of carrying out manually the important item of agitation, all have been added to the errors inherent in any serial dilution of particles in suspension. Although in spite of this the results are fairly regular and definite, one may not draw from them deductions upon the nature of bacteriophage further than that its behavior is in general what one would expect of any susceptible particulate substance suspended in fluid.

It should be said, however, that the graphs in figure 1 resemble the destruction rather of cultures of organisms than of chemicals or enzymes.

Initial and terminal deflections from the intermediate logarithmic descent are found characteristically in many disinfection curves,[24] while destruction of enzymes and other chemicals maintains the similarity to a monomolecular reaction throughout.[3]

All the values are of course higher by reason of the use of broth throughout, which absorbs the rays and tends to protect the bacteriophage. But, despite warnings like that of Zoeller[21] as to the impossibility of carrying out experiments on bacteriophage resistance in broth, we believe broth to be the correct diluent for this work. To use physiologic salt solution would introduce, unnecessarily, the added variable of progressive diminution in dissolved protein, and since one must start with broth, one can keep at least this factor (the absorption by dissolved protein) constant by continuing with the same concentration of protein in the substrate.

If a critical exposure, comparable to a critical temperature like 56.5 C., had been revealed by these tests, it would have somewhat favored the virus conception of bacteriophage. Any such period of exposure, however, below which lytic principle is quite unaffected and above which it rapidly succumbs, if it exists at all, must be less than 4 minutes (in broth), since this is the lowest exposure that has proved lethal in any of our work so far.

CONCLUSIONS

Within the limits of probable error in these experiments, the resistance to ultraviolet rays of this lytic principle is directly proportional to its concentration and appears to be a logarithmic function thereof. Destruction begins promptly and proceeds by a diminution in the number of active particles. The effect is not a photosensitization to heat. If irradiated tubes are observed without shaking, 18 hour incubation with B. coli D reveals regular differences in turbidity.

[24] Cohen, B.: J. Bact., 1922, 7, p. 183.

BACTERIOLOGIC STUDIES ON BIOLUMINESCENCE

1. CAUSE OF LUMINESCENCE IN THE FRESH WATER SHRIMP, XIPHOCARIDINA COMPRESSA (DE HAAN)

YOSHIWO YASAKI

From the Department of Bacterioolgy in the Tokyo Jikei-Kwai Medical College, Tokyo, Japan

One summer night, in 1914, Mr. Ushiyama, a teacher of the Suwa Middle School, first discovered luminous shrimps in Lake Suwa, a fresh water lake situated 100 miles from the nearest sea-shore at a height of about 1,000 meters from the sea level, in the prefecture of Nagano, Japan. Since then every year on summer nights he has observed many luminous shrimps swimming in the water—a beautiful sight. The cause of their luminescence, however, was not apparent.

That any luminous animals or bacteria, except the larvae of fire-flies, live in fresh water, was not known, so far as I was aware. Naturally, therefore, I was interested in the case reported by Ushiyama. In the summer of 1925 I caught 168 specimens of the luminous shrimps, investigated the cause of their luminescence, and found that the light production of the shrimps is due to infection by luminous bacteria,—pathogenic, not symbiotic.

The Shrimps.—The luminous shrimps live for a short time only, as will be shown below. The infection by luminous bacteria is not limited by the sex and age of the shrimps. The infection has no connection with the breeding season of the shrimp. The season varies; in general, it is during July and until September when the water temperature is about 23 to 30 C.

The shrimp is luminous from the end of tentacles to the legs, that is to say, the entire body glows. The light production is continuous and the color, a greenish white. The shrimps are intracellularly infected with luminous bacteria. The bacteria do not adhere to the surface of the shrimp's body. The light of the shrimp infected with luminous bacteria is seen through the transparent shell of the former and is so strong that one can read the letters of a book in a dark room, when he puts 20 or 30 of them in a test tube. The infected shrimps always die in a few hours after being taken, and also they die in the lake in a short time after being infected, but the light production continues for a few hours after death.

The Bacteria.—The writer could isolate luminous bacteria from the shrimp and could readily raise them in pure culture on ordinary agar medium (0.5% NaCl). The form of the luminous bacteria is quite similar to that of the Cholera vibrio. It is a slightly curved rod (0.5 by 3.0 microns) with one polar flagellum; stains with the ordinary anilin dyes, an aqueous solution of carbol fuchsin, diluted 1:10, being the most reliable staining agent; it is gram-negative, aerobic, and extremely motile. It grows well on ordinary solid mediums (agar gelatin, blood etc., P_H 7.2 to 8.0) and produces strong light. On agar the colonies are circular, pale blue-brown, moist, glistening, translucent and slightly
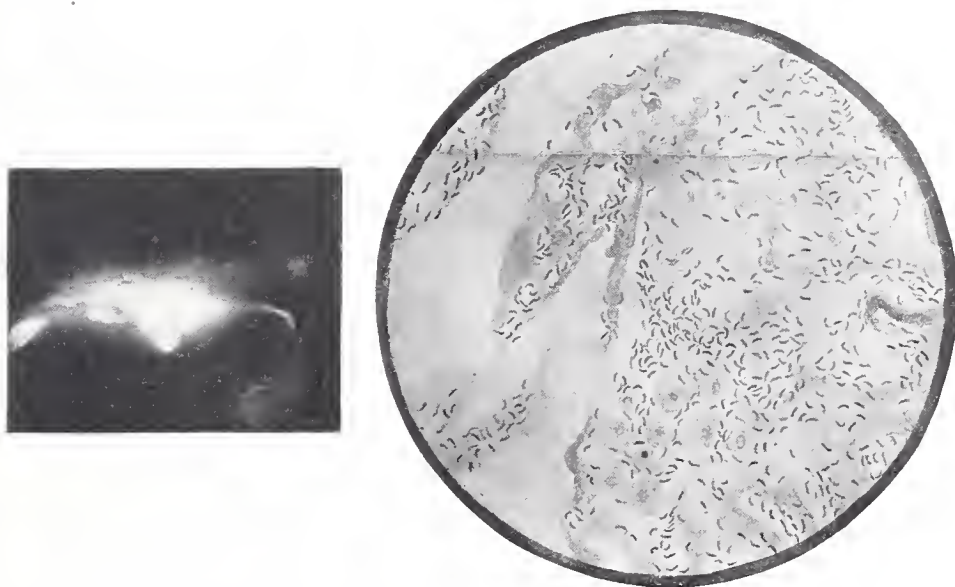
Fig. 1.—Female luminous shrimp, natural size.
Fig. 2.—Section of the green gland of a luminous shrimp.

raised. The vibrios grow on agar medium with no sodium chloride and produce light. They do not grow well on agar medium with 3% sodium chloride. It may be noted here that the water of Lake Suwa in summer contains chlorides in proportions of 8.0 to 15 Mg. per 1000 cc. In ordinary fluid mediums (bouillon, peptone water, milk, etc.) the bacteria produce very faint light seen only after violent shaking. Growth occurs at temperatures between 20 and 37 C., and is best at 37 C.; while photogenesis occurs at 25 to 30 C. These correspond to the water temperature of Lake Suwa in summer time. The light production in an incubator at 37 C. is feeble; it is also faint below 20 C.,

and disappears completely at 5 C. The development of the bacteria and
their light production, therefore, are not parallel. The culture mediums
must be weakly alkaline. The vibrio ferments dextrose, mannitol,
maltose, dextrin and amylum, but not saccharose, lactose, arabinose,
xylose, inulin, glycerol, dulcitol, rhamnose and salicin.

The bacteria die in 30 minutes at 50 C., while they are alive after
48 hours at — 6 C. They live for 5 minutes in a 1 : 5,000 HgCl solution,
for 8 hours in 1 :500 carbolic acid solution, for 10 minutes in 70%
alcohol, for 1 hour in 1 :500 formaldehyde solution. On culture mediums
they are viable for more than 30 days. Under certain conditions, they
become nonluminous; but they recover their capacity of light production
if they are injected into fresh water shrimps.

When dead animals are injected with the luminous bacteria, the
latter do not produce light. The bacteria are toxic for goldfish, carp,
gibles, loaches, pigeons, fowl, mice, guinea-pigs, besides shrimps, but
not for rabbits or man. They have an endotoxin: 0.02 mg. of the
bacteria kill a shrimp in 24 hours; 0.6 mgr. a fish, 7-8 cm. long; 0.7
mgr. a mouse of 15.0 Gm.; 2.0 mgr. a guinea-pig of 500 Gm.

In the serum of rabbits immunized with luminous bacteria, agglu-
tinins, precipitins, and complement-fixing substances were proved to be
present. The bacteria are immunologically distinguishable from the
Cholera vibrio and other water vibrios, and also from luminous vibrios
from sea-water.

The writer succeeded in making four kinds of shrimps luminous.
These were, Xiphocaridina compressa (de Haan), Palemon (Eupale-
mon) nipponensis (de Haan), Atyephira compressa (de Haan), Leander
pancidens (de Haan) and the larvae of Cybister joponica (Sharp).
They were given subcutaneous injections with a physiologic salt solution
suspension of luminous bacteria from an 18 hour agar culture; were
then placed in fresh water at 25 C., and became luminous within 5 to
10 hours. All these animals, however, died within 5 hours after
becoming luminous. Similar attempts with fish and other animals were
not successful, and all died. Healthy shrimps were also placed in fresh
water together with the luminous bacteria and they were found to be
luminous within one or two days. They died after being luminous for
two or three hours. It is clear, therefore, that the luminous bacteria
are pathogenic, not symbiotic in the body of the shrimp.

CONCLUSION

The luminosity of the shrimps in Lake Suwa is due to an infection by luminous bacteria. It may be said that the shrimps are suffering from a "light disease" caused by such infection.

No description of this form of luminous bacterium is found in the literature, and this is probably the first form found in fresh water. This luminous bacterium is named Microspira phosphoreum (Yasaki).

OBSERVATIONS ON THE ETIOLOGY OF MEASLES

Perrin H. Long and Agnes M. Cornwell

From the South Department and Thorndike Memorial Laboratory, Boston City Hospital

Recently Ferry and Fisher [1] have described a small, gram-positive, aerobic, green producing streptococcus which they isolated from the blood of measles patients in the early stages of the disease. In obtaining this organism 10 cc. of blood were added to flasks of Hibler medium and any resulting growth was plated on sheep's-blood agar. They state that this organism forms a soluble toxin, which on injection into horses or rabbits is capable of producing an immune serum showing antitoxic properties. Further, by using proper dilutions of the toxin, they have elaborated a skin test similar to the Schick test and Dick test, which can be used in determining individual susceptibility to measles.

In a series of papers published between 1918 and 1926 Tunnicliff [2] and her associates describe a gram-positive micrococcus which they believe to be the etiologic factor of measles. This organism, which can be cultivated only under strict anaerobic conditions in the first generation, grows, when transplanted upon suitable mediums, as an aerobic green-producing diplococcus or short chain streptococcus in subsequent generations. They have recovered this organism from the blood, sputum, nasopharyngeal secretions and the aural discharge of measles patients in the pre-eruptive stage and in the early period of the rash. They believe that they have produced a modified form of measles by inoculating susceptible animals with this organism. The serums of these inoculated animals possess the biological properties of immune sera. These workers have not as yet reproduced the disease in man.

Caronia [3] isolated an anaerobic gram-negative diplococcus from the blood, bone marrow, nasopharyngeal secretions and cerebrospinal fluid of measles patients using the Tarozzi-Noguchi culture technic. This organism is agglutinated in high dilution by the serum from convalescent measles patients. He states that he reproduced the disease in young

[1] J. Am. M. A., 1926, 86, p. 932.

[2] Ibid., 1917, 68, p. 1028; J. Infect. Dis., 1918, 22, p. 462; J. Am. M. A., 1918, 71, p. 104; J. Infect. Dis., 1919, 24, pp. 76, 181; Ibid, 1925, 37, p. 193; Tunnicliff, R., and Brown, M. W.: Ibid., 1918, 23, p. 572; Tunnicliff, R., and Moody, W. B.: Ibid., 1922, 31, p. 1; Tunnicliff, R., and Hoyne, A. L.: Ibid., 1926, 38, p. 48.

[3] Pediatria, 1923, 31, p. 801.

rabbits by inoculating them with this organism. He states that he was able to vaccinate children against measles by injecting killed strains of this organism, and claims to have reproduced the disease in a child by injecting living organisms.

In a carefully selected group of measles patients an attempt has been made to isolate a micrococcus from the blood stream. The method described by Ferry and Fisher has been followed in detail. The medium used by these investigators affords conditions of relative anaerobiosis and because of this fact it was thought that the micrococci described by Tunnicliff and Caronia might be isolated.

METHOD

Medium.—Hibler medium[4] was used throughout for blood culture work and was prepared as follows.

Reagent 1:—Ground beef liver tissue freed from fat, 500 Gm. and water, 1,000 Gm. are heated in flowing steam for 2 hours; peptone, 10 Gm. and sodium chloride, 5 Gm. are then added; the whole adjusted to Pʜ 8.2, and strained through gauze.

Reagent 2:—Beef brain freed from blood and membrane, 500 Gm. and water, 200 to 300 cc. to make a mixture of medium consistency, are heated in flowing steam for 2 hours. Then, using 250 cc. Erlenmeyer flasks, 120 cc. of reagent 1, and 60 cc. of reagent 2, are put into each flask, and autoclaved at 15 pounds pressure for 1 hour. The final Pʜ of medium after autoclaving was from 7.4 to 7.6.

Petri dishes containing 5% sheep blood beef infusion agar were used for plating, and 0.2% glucose beef infusion broth having Pʜ of 7.8 for toxin production.

Culture Technic.—In obtaining the blood cultures, 10 cc. of blood from the median cephalic vein was withdrawn sterilely and added immediately to a flask of Hibler medium which had been warmed to 37 C. The cultures were then incubated aerobically at 37.5 C., and were examined for growth every 24 hours for 3 days. Additional cultures from seven patients were incubated anaerobically in Brown[5] anaerobic jars for three days. If any growth was present, it was plated directly on sheep blood beef infusion agar and after incubation for 24 hours, identification of the organisms was undertaken.

RESULTS

In the selection of measles patients an attempt was made to choose those who were in the early stages of the disease (table 1).

The majority of these cases were the results of cross infections either in the open wards of the South Department or from the Pediatric wards of the Boston City Hospital. Forty-seven blood cultures were taken from 26 patients with measles. In 12 of these patients the blood cultures were taken during the pre-eruptive stage and later, after the rash had appeared. In all of the patients blood cultures were obtained during the early period of the rash.

[4] Personal communication from Dr. N. S. Ferry.
[5] J. Exper. Med., 1921, 33, p. 677; Ibid., 1922, 35, p. 467.

Growth was found in four flasks. In the blood cultures taken from two patients, pure growths of B. welchi were isolated. It was not clear whether these growths were the result of outside contamination or whether they resulted from spores in the culture medium that had resisted sterilzation. Control flasks of uninoculated culture medium

TABLE 1

Results of 47 Blood Cultures (Hibler Medium) from 26 Patients with Koplik's Spots

Patients		Dates of Cultures	Period of Disease	F. Temperature at Time of Culture	Course of Disease	Growth in 72 Hours
Number	Age					
One Culture from Each Patient						
1	5 years	5/ 5	No rash	100.2	Unknown	Negative
11	7 months to 19 years	....	Rash 3 to 18 hours	100.6 to 103.6	Mild (3), severe (6), m. severe (2)	Negative
2	3 years	4/23	Rash 6 hours	101	Severe	B. welchii
	4 years	6/13	Rash 6 hours	102.6	Moderately severe	B. welchii (aerobic) negative (anaerobic)
Two Cultures at Different Times, from Each Patient						
12	6 years	4/26	No rash	99.6	Severe	Negative
		4/27	Rash 6 hours	101.4		Negative
	20 years	4/26	No rash	100.6	Severe	Negative
		4/28	Rash 4 hours	103.2		Negative
	5 years	4/26	No rash	100	Severe	Negative
		4/27	Rash 4 hours	102		Negative
	5 years	4/27	No rash	101	Moderately severe	Negative
		4/29	Rash 8 hours	102.2		Negative
	3 years	4/29	No rash	100.6	Mild	Negative
		4/30	Rash 6 hours	101.8		Negative
	5 years	4/30	No rash	99.6	Mild	Negative
		5/ 1	Rash 6 hours	101.2		Negative
	2 years	4/30	No rash	100.6	Mild	Staphylococcus albus, Streptococcus viridans
		4/30	Rash 2 hours	101		Negative
	25 years	5/16	Rash 2 hours	101.6	Severe	Negative
		5/17	Rash 18 hours	102.6		Negative
	6 years	5/18	No rash	101.6	Severe	Staphylococcus albus
		5/18	Rash 4 hours	102		Negative
	3 years	5/25	No rash	99.8	Severe	Negative
		5/25	Rash 4 hours	101.2		Negative
	5 years	6/ 2	No rash	100.2	Moderately severe	Negative
		6/ 4	Rash 6 hours	102.2		Negative
	6 years	6/12	No rash	103	Severe	Negative
		6/12	Rash 1 hour	103.4		Negative

kept at incubator temperature for three days were invariably sterile. On the other hand as subsequently stated, an anaerobic cultivation of a culture from one of the patients yielded no growth of B. welchii. This would hardly be expected if the growth were due to outside contamination in view of the fact that the aerobic culture from the same syringe of blood yielded B. welchii. One blood culture yielded a pure culture of Staphylococcus albus.

A mixture of Staphylococcus albus and Streptococcus viridans was obtained in the first blood culture from one patient. The Streptococcus viridans was isolated in pure culture by picking individual colonies from a blood agar plate. It was then planted in 0.2% glucose beef infusion broth and incubated for six days in an attempt to produce toxin. Even very low dilutions (1:100) of the Berkefeld filtrate of the glucose broth culture produced no skin reactions in children with a negative history for measles.

From seven patients with measles separate blood cultures were incubated under both aerobic and anaerobic conditions. No growth was found in the flasks incubated anaerobically.

SUMMARY

An attempt was made to isolate from the blood of measles patients the toxin producing green streptococcus described.[1] Forty-seven blood cultures from 26 patients with measles were made in Hibler medium. Bacterial growth was found in four flasks at the end of seventy-two hours. These growths were considered to be the result of contamination. No toxin producing green streptococci were isolated.

THE INTRACELLULAR PROTEINS OF BACTERIA

2. THE GLOBULINS AS INDICATORS OF INTERSPECIES RELATIONSHIPS

CASPER I. NELSON

From the Department of Hygiene and Bacteriology of the University of Chicago

It is to be expected that in a group of organisms so inclusive as the colon-typhoid group some confusion would arise when closely allied species are concerned. Differentiation of members of the paratyphoid group from those of the enteritidis group, and differentiation within these subgroups has depended on the use of various technical factors which give results that are admittedly variable. Jordan [1] found that not all strains which agglutinated as B paratyphosus A fermented xylose, and within this species he found some strains which differed agglutinatively from normal paratyphosus A. He also found it of value to correlate differentiation by fermentation with host adaptability. But here again some of the results did not correlate with the results of agglutination. These various "departures from type" are indicative of the need of a more discriminative means of differentiation than we have available. It seems possible that a refinement of valuable methods now at hand may make it possible to classify the "departure from type" and to show that abnormalities are merely evidence of the fact that our arbitrary groupings may be related through liaison species.

Excepting for complement fixation, no technic has offered a more sensitive means of differentiating proteins of whatever source than the precipitin test. Species differences in plants and animals have been shown by this means. In bacteria, species differentiation has been accomplished by the precipitin test using various extracts of bacterial cells or their growth residues as antigens. The sensitization of animals with a single intracellular protein fraction from bacteria as precipitinogen, and the use of such a precipitating serum for the orientation of bacterial species have not yet been reported.

In a previous paper [2] a method is described for obtaining globulins from the cellular contents of seven members of the colon-typhoid group of bacteria. These globulins, when used as antigens for the production of antiserums in rabbits, produce precipitins which possess considerable

specificity. It was hoped that this specificity might offer a solution to the problem of antigen complexity and furnish a simple serological means to disentangle the biological confusion in the group. To determine whether this specificity is sufficiently marked to reveal the phylogenetic relationships that must exist between organisms similar in so many ways is the object of this phase of the work.

The isoelectric globulins obtained by the method described in the previous paper were suspended in 0.9% sterile salt solution, under thymol, and stored at refrigerator temperature until needed. About 3 cc. of precipitated globulin was available in each case. This was suspended in 10 cc. of sterile salt solution. One-fifth of this suspension was diluted to 20 cc. with sterile Ringer-Locke solution and then slowly dissolved by alkalinizing with N/10 NaOH. To this solution 0.5 cc. of standard phenolphthalein indicator was added directly and then just enough alkali at a time to maintain a faint pink color. Readjustments were made as soon as the neutralizing effect of the acid protein caused the color to fade. Heating to 45 C. hastened solution. Variations in the behavior of the various globulins is tabulated here:

Ringer's Solution with Globulin	Color of Final Solution, Phenolphthalein Present	State of Solution
B. typhosus................................... B. coli... B. enteritidis..................................	Deep pink	Clear
B. paratyphosus A*............................	Deep pink	Slightly opalescent
B. paratyphosus B.............................	Acid to phenolphthalein and brom thymol blue	Clear
B. dysenteriae Shiga...........................	Acid to phenolphthalein and brom thymol blue	Clear
B. dysenteriae Flexner*........................	Very pale pink. Alkaline to brom thymol blue	Opalescent

* In these two cases the opalescence was due to a second protein fraction which appeared only after several adjustments of reaction. The point of precipitation of this colloidal matter was within the solubility point of the globulin. Whether this condition represents a splitting of the globulin as a result of alkalinization and reaction or whether it represents a delayed separation of another fraction, is unknown.

Sensitization.—Rabbits were sensitized by four injections of 1 cc. amounts of globulin solution. Injections were made intraperitoneally, at intervals of four days. Eleven days after the last injection the animals were bled from the ear and the clear serum pipetted off from the clot.

Precipitin Tests.—Precipitin tests were made with fresh serum. The antigen, a clear solution of the globulin which had been centrifuged for 5 minutes at 800 to 1000 revolutions per minute, was maintained at constant concentration. 1 cc. amounts of progressive dilutions of serum were added in each case to a like amount of antigen. All tubes were incubated six to seven hours at 37 C. at which time the first readings were made. Subsequent check readings were made at the 24 and 48 hour periods.

A control was made with pooled normal rabbit serum. No evidence of precipitins for the bacterial globulins used was ever found either in pooled normal rabbit serum or in the serum of a single normal rabbit. Duplicate animals were not used in the tests. It is possible that experimental rabbits might show antibodies for one or more of the bacterial strains used because of accidental infections, but so far as circumstances permitted, this possibility was eliminated. All

the animals had been kept in their individual compartments from the time they were small and seemed to be in good condition.

In most cases where precipitation resulted, a ring formed at the interface of serum and antigen within a few minutes. For this reason all the tubes were agitated before being incubated.

In the case of some of the heterologous systems the precipitates were of a character quite different from that in homologous systems. In the latter the precipitates were heavy and compact, and difficult to disturb, but after being disturbed they settled readily again. The other precipitates were fine and formed a powdery deposit on the walls of the tubes.

It was found that two people could read the results independently and express them quantitatively with uniform agreement. The least amount of precipitate definitely visible was recorded as a trace (T). Other amounts were expressed comparatively by one or more plus signs. With due regard for the complications involved by so many elements it is believed that the following charts show that between the serums and the homologous antigens there is a degree of specificity which is typical; and in the heterologous systems this specificity exists in lesser degree. It is a commonly expressed opinion that this lesser heterologous specificity is in proportion to the molecular similarity of the heterologous protein to the type or homologous protein.

This work has shown that bacterial autolysates contain other protein fractions than those isolated. It is also very probable that the socalled bacterial globulin may represent more than one element which acts as precipitinogen. One may conceive of one or more of these elements as common to the entire related group. The precipitins produced against these elements common to the various related bacterial strains would be "group precipitins" and should appear in the lower dilutions of serum. Conversely, the higher dilutions of serum should contain the precipitins for those protein elements which are peculiar to the species. This simple hypothesis out of the many possible was the one adopted in the interpretation of these data.

Group relationships cannot be deduced from any single dilution of serum because of the possibility of simultaneous precipitates by group and type precipitins. Nor can any definite dilution be said to be the critical one. The whole series must be considered. The combination of group and species specificity must be taken as the index of phylogenetic relationship.

Mathematical Consideration of Specificity with Reference to Charts.—A visual comparison of so many values as are expressed in the charts is difficult and does not offer an exact measurement of specificity. A mathematical analysis, after the method suggested by Falk and Powdermaker[3] makes possible a comparison of the precipitin specificity of homologous systems with heterologous systems. It will be seen that the greater the heterogenity of the elements compared, the more favorable will be the case for specificity, and the more closely the elements compared are akin, the less favorable will be the comparison.

[3] J. Infect. Dis., 1925, 37, p. 514.
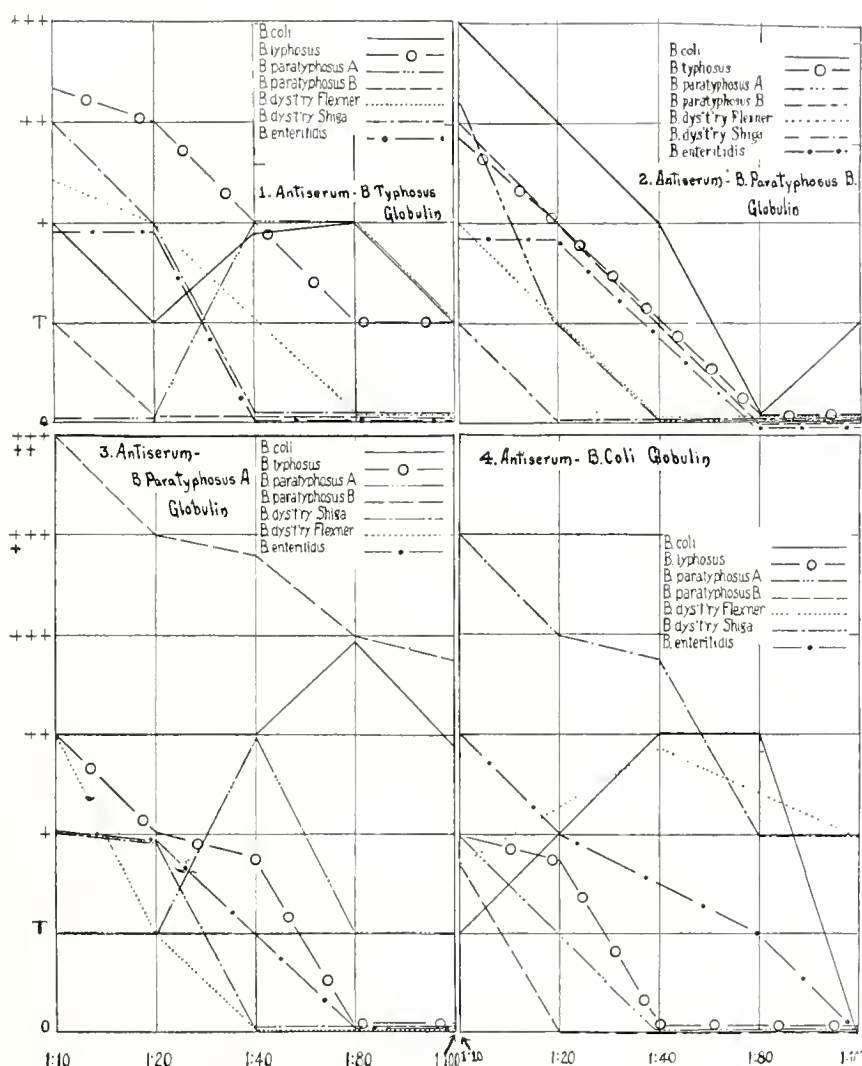
Fig. 1.—B. typhosus globulin is highly specific within the range of the dilutions. In this chart, as in all the others, B. coli assumes an intermediate position with reference to the serum. This seems to indicate its primitiveness. If the lower dilutions may be considered the "group range" B. typhosus shows strong group affinity for the dysentery group. Paratyphosus A approaches typhosus most closely in the "type range" of dilutions. The globulin of paratyphosus B acted sluggishly as an antigen, as all the charts indicate. Its resemblance to typhosus lies mostly in the low dilutions (group range).

Fig. 2.—The sluggishness of B. paratyphosus B globulin as an antigen is suggested by the low titers shown. In a general way the paratyphoids are grouped with typhosus and enteritidis. Paratyphosus A shows considerable dissimilarity to paratyphosus B except in the group range, while enteritidis shows much similarity. The dysenteries are well grouped and distinguished from the others. They are related through group affinity.

Fig. 3.—In this chart (and in 5) a heterologous antigen (paratyphosus B in both cases) appears more specific for the serum than the homologous antigen. This is not to be entirely credited without a repetition of the work. However, one may see in the results a certain coordination of the paratyphoid strains. One may also conclude that the globulin from paratyphoid B is readily precipitated by antiserums to the globulins of paratyphosus A and enteritidis, particularly the latter.

Fig. 4.—Here a close relationship between B. coli and the dysentery organisms is indicated, particularly in the higher dilutions. Charts 6 and 7 corroborate this statement fully. A sharp separation from the typhoid-paratyphoid group is to be seen. B. enteritidis seems to possess something of both groups.

For the sake of computation, arbitrary values have been assigned to the amounts of precipitin indicated in the charts. $T = 5$, $+ = 10$, $++ = 20$, etc. Attention has already been directed to the fact that the antigen paratyphosus B is the one marked exception to the rule of type specificity shown by these bacterial globulins. Its inclusion in the following tabulations reverses the balance in favor of homologous specificity which is otherwise quite uniform. For this reason a second computation is given omitting antigen paratyphosus B in titration with the antiserums in the globulins of paratyphosus A and B, enteritidis.

TABLE 1

COMPARISON OF HOMOLOGOUS AND HETEROLOGOUS SPECIFICITIES

Globulins		Dilution of Serum					All 5 Dilutions		Last 3 Dilutions	
		1:10	1:20	1:40	1:80	1:100	Average	% Basis	Average	% Basis
B. typhosus	Homologous	24	20	10	5	5	12.8	100.0	6.6	100.0
	Heterologous	9.5	5.3	4.6	3.3	1.6	4.8	34.4	3.2	48.0
B. paratyphosus A	Homologous	5	5	20	5	5	8.0	100.0	10.0	100.0
	Heterologous	21.3	15	12	10	7	13.0	162.5	10.0	100.0*
B. paratyphosus B	Homologous	20	10	5	0	0	7.0	100.0	1.7	100.0
	Heterologous	15.5	8	4	0	1	5.7	81.3	1.7	100.0
B. enteritidis	Homologous	30	20	8	8	5	14.2	100.0	7.0	100.0
	Heterologous	17	18	12.5	16.6	12	15.2	107.0	13.7	196.0*
B. coli	Homologous	5	10	20	20	0	11.0	100.0	13.3	100.0
	Heterologous	15.2	11	5	5	3.3	7.9	71.8	2.8	21.0
B. Shiga	Homologous	50	40	40	30	30	38.0	100.0	33.3	100.0
	Heterologous	24.3	20.5	13	6.6	3	13.5	35.5	7.5	22.5
B. Flexner	Homologous	30	30	20	18	5	20.6	100.0	14.3	100.0
	Heterologous	18.5	16	4.6	2.5	0	8.3	40.3	2.4	16.7
B. paratyphosus A	Homologous	5	5	20	5	5	8.0	100.0	10.0	100.0
	Heterologous	11.6	10	6.6	6	3	7.4	92.5	5.2	52.0†
B. enteritidis	Homologous	30	20	8	8	5	14.2	100.0	7.0	100.0
	Heterologous	12.4	6	6	8.2	4.4	7.2	50.7	6.2	88.5†

* Averages in which B. paratyphosus B globulin is included.
† Averages eliminating B. paratyphosus B globulin.

In order to determine whether group specificity is indicated by this arithmetical expression of quantitative titer values, a comparison of the averages of homologous and heterologous values at each serum dilution is given:

TABLE 2

RATIOS OF PRECIPITIN TITER ACCORDING TO SERUM DILUTIONS

	Dilutions				
	1:10	1:20	1:40	1:80	
Average, homologous	23.4	18	17.55	12.3	7.1
Average, heterologous	15.3	11	6.26	4.5	2.6
Ratio	100:65.2	100:61.1	100:35.6	100:36.5	100-36.6

The preceding tabulation of precipitin values of the serum dilutions with both homologous and heterologous antigens demonstrates relationships which would otherwise remain undetected. Expressed as a graph (fig. 8) it is seen that the titers with homologous antigen describe a

curve whose regularity is interrupted at the dilution 1/40. It is a composite curve and represents two factors in the specificity of the homologous antigens and sera. The above ratios bring out this fact even more clearly. The first two dilutions are concerned with "group specificity" and the last three with "type specificity." On the other hand the titers of the serums with heterologous antigens describe a simple linear curve such as is usually associated with immune serums. The values in this curve are in simple linear proportion.

TABLE 3

Comparison of Homologous and Heterologous Antigen Precipitabilities

Globulins		Dilutions					All 5 Dilutions		Last 3 Dilutions	
		1:10	1:20	1:40	1:80	1:100	Average	%	Average	%
B. typhosus	Homologous	24	20	10	5	5	14.8	100.0	7.0	100.0
	Heterologous	14.3	10.6	4.6	2.16	0	6.33	42.7	2.25	31.1
B. paratyphosus A	Homologous	5	5	20	5	5	8.0	100.0	10.0	100.0
	Heterologous	14	6.6	4.3	2.5	0.83	5.64	70.0	2.54	25.4
B. paratyphosus B*	Homologous	20	10	5	0	0	7.00	100.0	1.66	100.0
	Heterologous	11.75	7	4	0.83	0.83	4.88	69.9	1.88	88.3
B. enteritidis	Homologous	30	20	8	8	5	14.2	100.0	7.0	100.0
	Heterologous	17	11	7	2.5	0	7.5	52.8	3.16	45.1
B. coli	Homologous	5	10	20	20	0	11.0	100.0	13.3	100.0
	Heterologous	21	18.3	13.5	13.3	7	15.0	131.0	11.26	84.6
B. dysenteriae Shiga	Homologous	50	40	40	30	30	38.0	100.0	33.3	100.0
	Heterologous	22.5	12	6	3.3	2.5	9.26	24.4	3.9	11.7
B. dysenteriae Flexner	Homologous	30	30	20	18	5	20.6	100.0	14.3	100.0
	Heterologous	14.3	10.3	8	5.5	4.3	8.48	41.1	5.9	41.3

* Values of B. paratyphosis B globulin precipitated by serums to B. enteritidis and B. paratyphosus A antigen omitted.

TABLE 4

Ratios of Precipitability According to Serum Dilutions

	Dilutions				
	1:10	1:20	1:40	1:80	1:100
Average, homologous	23.43	19.3	17.6	12.3	7.14
Average, heterologous	16.41	9.4	6.86	4.3	2.21
Ratio	100:70.0	100:48.6	100:38.9	100:34.9	100:30.95

A comparison of these ratios shows that the precipitability of the heterologous and homologous antigens decreases in direct proportion to the increasing dilution of the serums. In such a graph there is naturally no evidence of the grouping of these values such as is seen in the tabulation of precipitin titers above. The curves described (fig. 9) are quite similar and yield to an interpretation similar to that for the previous ones.
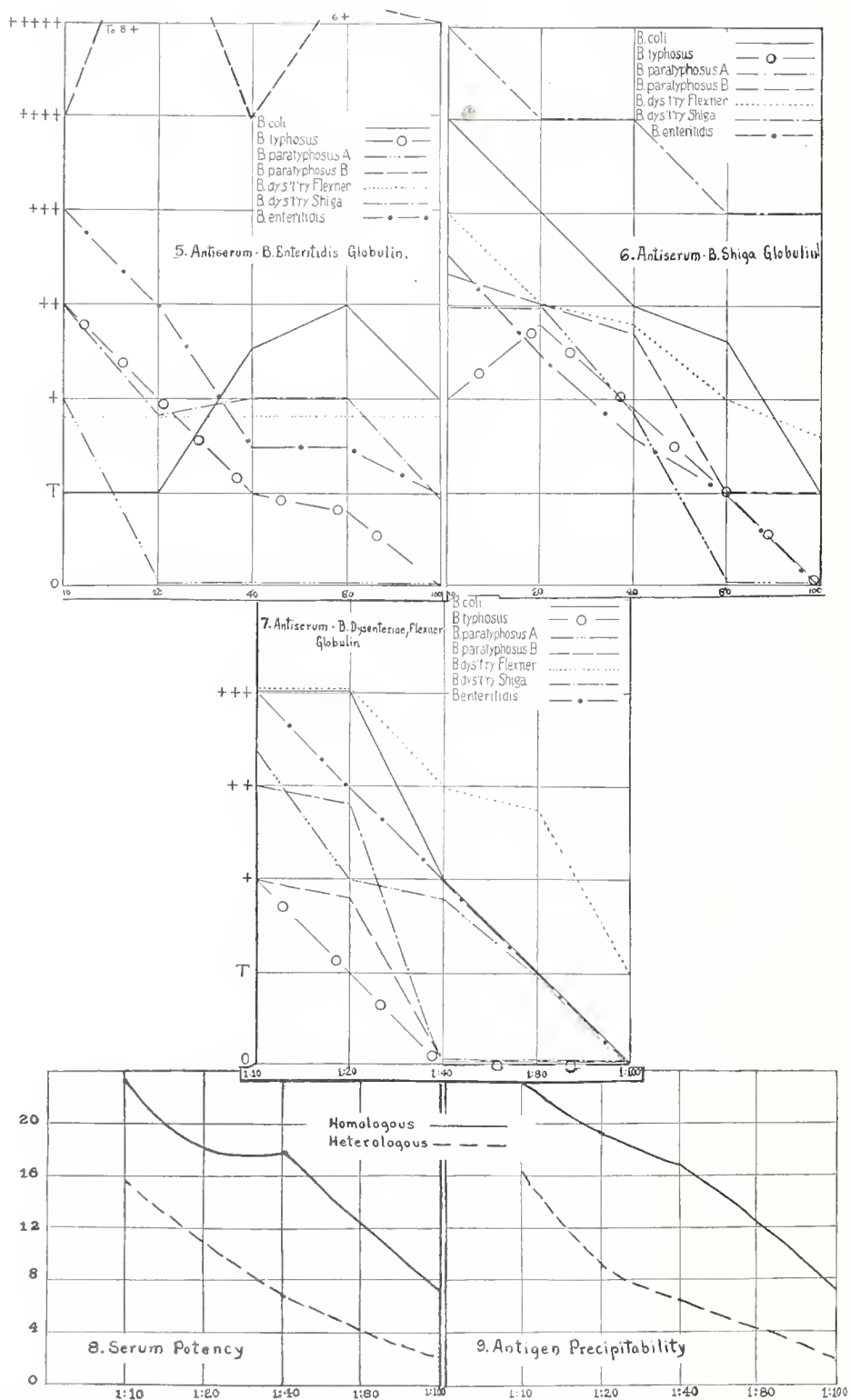
Fig. 5.—A physico-chemical explanation would better fit the occurrence of such a marked heterologous precipitation as occurs here in the case of paratyphosus B. Aside from that, the globulin from B. enteritidis shows fair specificity in the group range. Its dissimilarity to paratyphosus A is well indicated. (The Flexner globulin was precipitated by acidity which prevailed in that particular series.)

Figs. 6 and 7.—The orderliness of these two charts leave little to be said beyond what has already been pointed out. The serologic difference in the two dysentery strains, as well as the linkage to B. coli, is marked.

Figs. 8 and 9.—Serum precipitin titers with homologous and heterologous antigens according to serum dilutions (see text).

From the preceding data is obtained the following sequence of decreasing serum potency and antigen precipitability.

AGGLUTINATION REACTIONS OF THE ANTIGLOBULIN SERUMS

Serum Potency		Antigen Precipitability	
Homologous	Homologous	Heterologous	Heterologous
B. dysenteriae Shiga	B. dysenteriae Shiga	B. dysenteriae Shiga	B. coli
B. dysenteriae Flexner	B. dysenteriae Flexner	B. dysenteriae Flexner	B. paratyphosus B
B. enteritidis	B. coli	B. paratyphosus A	B. enteritidis
B. typhosus	B. paratyphosus A	B. enteritidis	B. typhosus
B. coli	B. enteritidis	B. typhosus	B. dysenteriae Flexner
B. paratyphosus A	B. paratyphosus B	B. coli	B. paratyphosus A
B. paratyphosus B	B. typhosus	B. paratyphosus B	B. dysenteriae Shiga

A uniform grouping of the dysentery organisms is seen in these sequences. There is also a grouping of the typhoid and paratyphoid organisms. B. coli and B. enteritidis occupy intermediate places. B. paratyphosus B and B. enteritidis are somewhat differentiated from the other strains.

The difference between precipitation and agglutination reactions lies in the type of antigen used. The following tests were made to determine whether the specificity that holds for the simpler, unformed antigen, the bacterial globulin, would hold in the same degree and manner for the formed elements, the cells.

Cultures of the bacteria grown for eighteen hours on nonprotein synthetic agar were suspended in very weak saline solution (0.1%). The suspensions were centrifugated at low speed for a minute or two to eliminate clumps and debris. Preliminary microscopic agglutination tests indicated the range of serum dilutions necessary, i. e., 1/20 to 1/2000. The tubes with bacteria and serum were prepared in the usual manner, incubated at 37 C., and several observations made. The first reading was made after eight hours' incubation. The results of later readings were not significantly different.

The flocculations were much more definite than those usually obtained with antibacterial serums. Spontaneous agglutination occurred with the dysentery bacilli, but this was largely avoided by early readings.

A number of physical factors are concerned in the agglutination of bacterial cells. Ordinarily, these factors are not well governed. If the production of an antiserum to such a comparatively simple antigen as a protein fraction gives an antibody of any selective power, then the results of the titration of a less simple antigen, the whole cell, with that anti-serum should indicate diminished selectivity. The data in figures 10 and 11 indicate that this is the case. Group specificity is clear. The troughs

and crests of the curves agree within the typhoid-paratyphoid group, also within the dysentery group. B. enteritidis and B. coli agree only in parts with each group and so cannot be placed in either. For purposes of comparison, the curves for B. coli and B. enteritidis are placed on both charts.

Agglutination tests with antiserums to bacterial globulins do not lend themselves to quantitative measurements of the masses thrown down. In general, it can be stated that the finer type specificities are hidden in the general group specificities. This is explainable on the basis of the more complex antigen used. It is further possible that the usual bacterial suspension contains an appreciable amount of autolyzed bacterial substance also giving results which are a mixture of two or more systems.
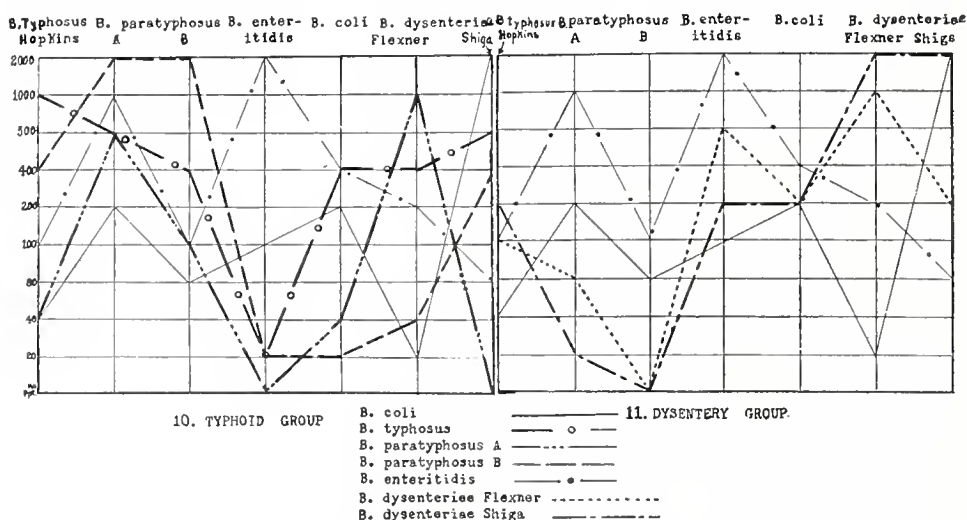
Figs. 10 and 11.—Agglutination titers, indicating diminished selectivity of globulin antiserums for the whole bacteria.

THE DIAGNOSTIC VALUE OF AN ANTISERUM PRODUCED WITH BACTERIAL GLOBULIN

At one time in the progress of this work the culture of B. paratyphosus B was lost. In lieu of it another culture which was at hand was used as a source of globulin. A check-up of the fermentative characteristics of this second culture aroused doubt that it was B. paratyphosus B. A new culture of B. paratyphosus B, Aertrycke type, was obtained from the collection of Dr. E. O. Jordan of this laboratory. Globulin and the corresponding antiserum were prepared from this culture. A titration of the doubtful globulin against the several known antiglobulin sera indicates as is shown by the following tabulation that the unknown

organism corresponds most closely to B enteritidis. The cultural characteristics of the unknown organism led to the same conclusion.

UNKNOWN GLOBULIN
Serum Dilution

Antiserum	1/10	1/20	1/40	1/80	1/100
B. coli	0	0	0	0	0
B. dysenteriae Shiga	0	0	0	0	0
B. dysenteriae Flexner	0	0	0	0	0
B. typhosus	0	0	0	0	0
B. enteritidis	++	+	+	T	0
B. paratyphosus B	T	T	0	0	0
B. paratyphosus A	0	0	0	0	0

The group specificity of the globulin of the colon-typhoid group extends also to more distant members of the group. A suspension of B. alkaligines, when titrated against the several antisera showed a moderate heterologous specificity with B. paratyphosus B (1/200), B. coli and B. enteritidis, in the order named.

RELATION OF TYPHOID GLOBULIN ANTISERUM TO HIGHLY AGGLUTINABLE STRAINS

The well known tendency of the Rawlings strain of B. typhosus to agglutinate in high titer was maintained against the antiserum produced against globulin from the Hopkins strain of B. typhosus used in this work. The titer with the Hopkins strain was 1/1000, but with the Rawlings strain (and also with the Barrymore strain) the titer was 1/12,500 which indicates that high agglutinability is a characteristic of the organisms affected rather than of the agglutinating serum.

DISCUSSION

No effort has been made to defend the case of the globulins as the biologic specific principle of the bacterial species against the case of the carbohydrate specific principle which is advocated by some authors.[4, 5, 6] It is clear that the specific principle whatever it is is associated with the globulin fraction.

An analysis of the data shows that in the globulin there is a double specificity, one for the group, sufficiently marked to distinguish group from group, and one for the type, sufficiently marked to differentiate members within a group.

[4] Avery, O. T., and Morgan, H. J.: Jour. Exper. Med., 1925, 43, pp. 347-353.
[5] Heidelberger and Avery: Jour. Exper. Med., 1923, 38, pp. 73-79.
[6] Ottenberg, R.: Proc. Soc. Exp. Biol., 1923, 21, 14. Ottenberg, R., and Stenbuck, F.: Proc. Soc. Exp. Biol., 1923, 21, pp. 303-307.

Finer determinations can be made by means of the precipitin test by using a simplified antigen, such as the globulin, than by using the corresponding "formed" antigen. (The identity of precipitin and agglutinin is assumed). It is suggested that some of the variable results obtained in the serological determination of bacterial species may be due to this limitation in the use of agglutination because of the fact that bacterial suspensions may represent both "formed" elements and intracellular proteins.

The methods described may be used for the determination of bacterial identity, as a corollary to the cultural and biochemical determination.

Globulin antigens may be prepared in quantity for sensitization purposes and stored until needed. They can then be used to produce specific antiserums of high titer, and without such injury to the animal as comes from the injection of suspensions of pathogenic organisms.

SUMMARY

Globulins obtained from seven species of the colon-typhoid group of bacteria were effective antigens for the production of precipitins in rabbits. These globulins are highly specific for their homologous antiserums. They also possess a specificity for heterologous serums which is quantitatively proportional to the genetic relationship which the heterologous derivative bears to the homologous one. The data of complete direct and cross precipitin titrations of the bacterial globulins and their antiserums, when analyzed objectively and statistically, yield a sequence of interspecies relationship about as follows:

Group A   1. B. typhosus
          2. B. paratyphosus A
Group B   3. B. paratyphosus B (Aertrycke type)
          4. B. enteritidis
Group C   5. B. coli
Group D   6. B. dysenteriae Flexner
          7. B. dysenteriae Shiga

This is quite in agreement with the latest opinions concerning the host distribution of this group.[7] Two very distinct groups of organisms are recognized, the dysentery group and the typhoid-paratyphoid A group. There is a third less well defined group, the enteritidis-paratyphoid B group. A fourth group is represented by B. coli which is linked in a fundamental way with all the other organisms and particularly with the dysentery group. B. coli, genetically speaking, is a stem organism.

[7] Jordan, E. O.: J. Infect. Dis., 1925, 36, p. 309.

EFFECT OF GENTIAN VIOLET AND MERCURO-CHROME ON ENDOMYCES ALBICANS

WITH SPECIAL REFERENCE TO THE MYCELIAL FORM

Esther Bridgman Clark

From the Division of Pediatrics, Stanford University Medical School, San Francisco

Because of the excellent results obtained with 1% gentian violet in the treatment of thrush in infants, as reported by Faber and Dickey,[1] a series of experiments were carried out to determine the germicidal effect of various dilutions of gentian violet on Endomyces albicans in cultures. Churchman[2] stroked divided gentian violet plates with Oïdium albicans and noted complete inhibition of growth at 1:100,000 dilution of the dye; he also states that Oïdium albicans stained with gentian violet failed to grow. However, he does not state whether his cultures were growing in the yeast or mycelial form. As thrush occurs in the mouths of infants the mycelial form predominates, so it would seem to be of some importance to test the effect of gentian violet on Endomyces albicans (Oïdium albicans) growing in the mycelial form. On ordinary mediums transplants of thrush usually show a predominating growth of yeast forms. Draper[3] obtained a good growth of mycelia in a carrot infusion medium, made by soaking ground up carrots in sterile distilled water at 10 C. for 48 hours, then filtering and autoclaving.

In these experiments a similar medium was used and agar plates were made, adding 10% agar to the carrot infusion. On this medium mycelial forms would soon appear, and in old cultures would be very numerous. Divided plates were made by pouring one half with plain carrot agar and the other half with carrot agar and gentian violet. Inoculation of the plate was done by stroking across both halves, the plain carrot agar serving as a control on each plate. The cultures were obtained from mouth lesions which revealed mycelia and spores in smears, and clinically were diagnosed thrush. They were grown in the carrot infusion and plated out on carrot agar plates until pure cultures were obtained. The results of the experiments are contained in table 1. In all cases smears from the plates were examined microscopically and found to contain yeast and mycelial forms. In every case there was a good growth on the half of the plate serving as control.

[1] J. Am. M. A., 1925, 85, p. 900.
[2] J. Exper. Med., 1921, 33, p. 569.
[3] J. Infect. Dis., 1924, 34, p. 631.

TABLE 1

RESULTS OF CULTURE OF THE MYCELIAL FORMS OF ENDOMYCES ALBICANS ON CARROT AGAR PLATES WITH VARIOUS DILUTIONS OF GENTIAN VIOLET AND MERCUROCHROME

Dilution of Dye in Carrot Agar Plate	Subcultures on Gentian Violet Plates			Subcultures on Mercurochrome Plates from Sabaraud's Medium
	From Carrot Infusion Culture	From Carrot Agar Plate	From Sabaraud's Medium	
1:100 to 1:25,000	No growth	No growth	....	Good growth 1:10,000
1:50,000	Marked inhibition	No growth	....	....
1:75,000	Marked inhibition	No growth	....	....
1:100,000	Definite inhibition	No growth	No growth	Good growth
1:200,000	....	Marked inhibition	Marked inhibition	Good growth
1:300,000	....	Marked inhibition	Inhibition	Good growth
1:400,000	....	....	Slight inhibition	Good growth
1:500,000	Good growth	....	Good growth	Good growth
1:1,000,000	Good growth	....	....	....

CONCLUSIONS

Gentian violet completely inhibits the growth of the mycelial and yeast forms of endomyces albicans in dilutions up to 1:100,000, and inhibits to some extent in dilutions to 1:400,000.

A dilution of 1:10,000 of mercurochrome causes no inhibition in the growth of the mycelia of yeast forms of endomyces albicans.

FOOD ACCESSORY SUBSTANCES (VITAMINS) AND TUBERCLE BACILLI

1. DO TUBERCLE BACILLI CONTAIN OR PRODUCE GROWTH PROMOTING SUBSTANCES?

Nao Uyei

From the Research Department, National Jewish Hospital at Denver, Colorado

Since the discovery of food accessory substances or vitamins by the pioneer workers in this field, an enormous amount of work has been done in the study of the nutritional problems among the higher animals. Similar studies have extended not only to the higher forms of plants, but also to the microorganisms as well. The necessity of such substances for the maintenance and growth of higher animals is now generally recognized, but regarding their significance in the nutrition and growth of plants and microorganisms opinion is divided.

In the case of higher plants Bottomley[1] and Mockenridge and others[2] recognize the necessity of certain growth stimulating substances or auximones, while, on the other hand, Lumiére,[3] and Clark and Roller,[4] maintain that plants need no vitamin or vitamin-like substances for normal growth.

Regarding the necessity of food accessory substances for the growth of yeast, there exists a diversity of opinions. Bachman, Williams, Swoboda, Eddy, Stevenson, Funk and Dubin,[5] Robertson and Davis[6] consider the necessity of a growth promoting substance or substances for yeast, while Souza, McCollum, MacDonald and McCollum, Fulmer, Nelson and Sherwood,[5] and Tanner[7] are inclined to believe that there is no necessity for assuming growth stimulating substances or bios essential to the normal growth of yeast plants.

In a study of the relation of food accessory substances to bacterial growth, a number of investigators report on the necessity of vitamins for their growth. It suffices to mention briefly some of the important studies done. Lloyd[8] first stimulated interest along this line by presenting evidence that primary cultures of meningococcus are impossible of accomplishment without the presence of a growth accessory factor in the culture medium. Pacini and Russel[9] detected the presence of vitamin in the typhoid bacillus, Davis,[10] in a study of the growth of hemophilic bacilli, comes to the conclusion that they require at least two growth promoting substances, one of the nature of vitamin B and the other

[1] Proc. Roy Soc., 1914, 88B, p. 237; 1915, 89B, p. 102.
[2] Mockenridge: Ibid., 1917, 89B, p. 508; Rosenheim: Biochem. J., 1917, 11, p. 7.
[3] Compt. rend. Acad. d. sc., 1920, 171, p. 271.
[4] Soil Sc., 1924, 17, p. 193.
[5] For bibliography of these works see Sherman and Smith: The Vitamines, 1922; Funk and Dubin: The Vitamines, 1922; Ellis and McLeod: Vital Factors of Foods, 1922; Tanner: Chemical Rev., 1925, 1, p. 397.
[6] J. Infect. Dis., 1923, 32, p. 153.
[7] J. Bact , 1926, 11, p. 45.
[8] J. Path. & Bact., 1916, 21, p. 113; Brit. M. J., 1916, 2, p. 743.
[9] J. Biol. Chem., 1918, 34, p. 43.
[10] J. Infect. Dis., 1917, 21, p. 21, p. 392; 1921, 29, p. 171.

of greater heat stability which was originally thought to be identical with the hemoglobin of the blood. The work of Shearer[11] is interesting from a pathological view point in that cerebrospinal fluid stimulated the growth of the meningococcus more than blood or nasal mucus. Kligler[12] obtained favorable action of water soluble vitamin from animal tissues on the growth of several pathogenic bacteria.

In spite of the fact that a great deal of work has been done on the influence of vitamins on the growth of other bacteria, a review of the literature reveals very little work on the relation of the food accessory substances or vitamins to the growth of tubercle bacilli. Corper[13] studied the difference in the rate of growth of recently isolated tubercle bacilli and old laboratory cultures on mediums containing blood or various animal tissues, but did not discuss the results from the standpoint of vitamin requirement for bacterial growth. Cunningham[14] tried to obtain evidence for the presence of vitamins A and B in the tubercle bacillus but the results were negative. Long,[15] mentions the absence of stimulating action of commercial yeast vitamin preparations on the market on the growth of tubercle bacilli, but did not describe his method of study or give the data obtained.

The present article and the one following are reports of an investigation on the relation of food accessory factors or vitamins to the growth of the tubercle bacillus. It was hoped to throw some light on the interpretation of experimental tuberculosis with reference to vitamins and perhaps also on problems concerned with the diet and nutrition of tuberculous patients.

Pasteur and others noted that when an artificial culture medium is inoculated with a very small amount of yeast, the yeast would not grow. Wildiers [16] interpreted this fact in assuming that the growth of the yeast is only possible in an artificial culture medium after the generation of an essential nutrient material called by him "bios" through the death and autolysis of yeast cells. The necessity of such a substance for the growth of yeast has led to a controversy between many investigators cited earlier in this paper. Since it is well known that tubercle bacilli and many other bacteria do not grow when seeded too thinly on artificial culture mediums this was chosen as the introductory part for a study of the relation of the food accessary factors to the growth of the tubercle bacillus.

Methods.—In order to study the vitamin requirements for the tubercle bacillus, it is essential to know the smallest amount of seeding necessary to obtain a successful growth of the tubercle bacilli. With this established, the presence or absence of a growth stimulant in a substance under consideration can be

11 Lancet, 1917, 1, p. 59.
12 J. Exper. Med., 1919, 30, p. 31.
13 Am. Rev. Tuberc., 1919, 3, p. 461.
14 Ibid., 1924, 9, p. 487.
15 Ibid., 1922, 5, p. 713.
16 La Cellule, 1901, 18, p. 313.

determined by adding this substance to a standard control medium, and noting acceleration or retardation of growth as the case may be. Uniformity of method in seeding can best be attained by using the suspension method of inoculating the culture medium. For this purpose, a weighed quantity of young actively growing tubercle bacilli were thoroughly ground to a uniform suspension in a graduated centrifuge tube using a definite volume of physiologic salt solution. From this stock solution, various dilutions were made with sterile salt solution and the culture tube containing the standard control medium was seeded uniformly by using just enough of the suspension to form a thin moist film of bacilli over the slanted surface of the medium. Long's medium[17] was modified so as to contain 2½ per cent of agar and used as the standard control medium. The composition of the medium is given here:

	Gm.		Gm.
Asparagin	5.00	Magnesium sulphate	1.00
Ammonium citrate	5.00	Ferrous ammonium citrate	0.05
Potassium acid phosphate	3.00	Glycerol	50.00
Sodium carbonate	3.00	Agar	25.00
Sodium chloride	2.00	Water	1000.00

Both avirulent human and bovine strains of tubercle bacilli were used in these experiments. The growth of the cultures were noted weekly.

TABLE 1

The Growth of Tubercle Bacilli as Affected by Dilution in Suspension

Concentration of the Suspension of Tubercle Bacilli	Type of Bacilli				
	Human Avirulent			Bovine D	
	2 Weeks	4 Weeks	8 Weeks	3 Weeks	5 Weeks
A (100 mg. per cc.)	+	+++	++++	+	++
	±	±	±	±	++
	±	+++	++++	+	++
	+	+++	++++	+	+
B (50 mg. per cc.)	±	±	++	±	+
	±	+	+++	±	+
	±	+	+++	±	+
	±	+	+++	±	±
C (10 mg. per cc.)	0	0	0	0	0
	0	0	⊕	0	0
	0	0	0	0	0
	0	0	⊕	0	0
D (5 mg. per cc.)	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0

The rate of growth in this article is graded from 0 for no growth to ++++ a profuse, excellent growth. ⊕ indicates that the growth occurred in only one or two spots probably due to lumps of bacilli which were not well divided in making the suspension.

From table 1, it is evident that tubercle bacilli when seeded as a suspension containing 10 mg. or less per cc. on Long's medium are incapable of growth, while in concentrations of 50 mg. or above, per cc.

[17] Tubercle, 1924, 6, p. 128.

they are capable of multiplying and growing. Since Corper [18] found that tubercle bacilli in an amount as small as 0.000,001 mg. were still capable of producing an active tuberculosis in the guinea-pig after subcutaneous injection, the failure of the growth of the tubercle bacilli in a suspension containing 10 mg. per ml., cannot be considered to be due necessarily to the presence of dead bacilli in the original suspension. It seemed interesting, therefore, to determine whether mediums containing various complex organic nutrient substances, such as Dorset's egg medium, Petroff's gentian violet egg medium and nutrient 5% glycerol-agar mediums commonly used for growing tubercle bacilli, would be capable of giving growth when seeded with very fine suspensions (table 2).

The results recorded in tables 1 and 2 will be given full consideration in the discussion. They are, in general, in harmony with the view that tubercle bacilli either contain within their cells, or produce by virtue of their own metabolic activities, or elaborate after death and by autolysis a growth promoting substance or substances. In order to throw light on the question whether tubercle bacilli contain within their cells growth promoting substances, an acetone extract and a water extract of tubercle bacilli were prepared and the influence of these extracts on the growth of the bacilli was studied. In order to learn whether the growth of tubercle bacilli is influenced by their metabolic products or by their products of bacterial autolysis, a tuberculin preparation which contains both the metabolic products and the products of bacterial autolysis, was prepared and its effect on the growth of the bacilli observed.

Acetone Extract.—To prepare the acetone extract, a weighed quantity of fresh growing tubercle bacilli was extracted with pure acetone in a Soxhlet's apparatus. The time of extraction varied from three hours in one sample to forty-eight hours in another. The extract was concentrated to 10 cc. and from this solution various dilutions were made using acetone as the solvent. , One cc. each of the acetone solution was introduced into a culture tube containing 9 cc. of Long's medium, while the culture tubes for the control contained 1 cc. of pure acetone added to the same amount of the medium. These tubes were then sterilized, and the acetone driven off by heating in an Arnold sterilizer at 95 C. for one hour each on two successive days. These tubes were seeded with a suspension containing 75 mg. of tubercle bacilli per cc. In later experiments, a suspension containing 50 mg. per cc. was used. As a result of later studies 30 mg. per cc. amount of tubercle bacilli seeded on Long's medium was found to result in a slow growing culture which on account of its thin and slow growth was found to be excellent for use in the study of the effect of the growth

[18] J. Infect. Dis., 1918, 23, p. 493.

TABLE 2

The Growth of Human Tubercle Bacilli in One to Six Weeks on Long's Medium as Compared with Growth on Common Laboratory Mediums

Concentration of Bacilli in the Suspension

	50 mg. per cc.						25 mg. per cc.						10 mg. per cc.						5 mg. per cc.						1 mg. per cc.					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Long's Synthetic Nonprotein Medium	?	±	±+	++	++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	?	±+	++	++	++	......	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dorset's Egg Medium	?	++	++	+++	+++	+++	?	++	+±	++	+++	+++	?	±±	++	++	++	+++	?	±±+	+±	++	+±	+++	?	0	0	0	0	0
	?	++	++	+++	+++	+++	?	++	+±	+++	+++	+++	?	+±	++	++	++	+++	?	±±+	±±	++	–	+++	?	0	++	0	–	–
Petroff's Gentian-Violet Egg Medium	?	++	++	+++	+++	+++	?	±±+	++	++	++	+++	?	±±+	±±	++	++	±+	?	±±+	++	++	++	++	?	±±+	±±+	++	++	++
	?	++	++	+++	+++	+++	?	+±	++	++	++	+++	?	±+	±±+	++	++	+++	?	±±+	++	++	++	+++	?	±±+	±±+	++	++	++
5% Glycerol Nutrient Agar Medium	?	++	++	++	++	+++	?	++	++	++	++	++	?	±±+	++	++	++	++	?	?	±±+	±±+	±±+	++	?	?	±±+	±±+	±±+	++
	?	++	++	++	++	+++	?	+±	++	++	++	++	?	±±+	+±	++	++	+++	?	?	±±	±±+	±±+	++	?	?	±±+	±±+	±±+	++

stimulants on the tubercle bacilli. The seeded cultures were kept in an incubator at 37 C. and the growth of the culture was examined every week, but the results proved to be indecisive.

Water Extract.—To prepare the water extract of tubercle bacilli the residue from the acetone extract was first dried in warm air at about 40 C., after which it was extracted by means of hot water at varying intervals from three to forty-eight hours. The extract was concentrated in vacuum and made up to 10 cc. with water. The remainder of the procedure was identical to that for the acetone extract, except that the solvent was distilled water instead of acetone.

Six experiments on the effect of the water extract on the growth of tubercle bacilli, using different strains of bacilli resulted in a slight stimulating effect in some cultures while in others there occurred a retardation, but the majority showed no effect of the watery extract upon the growth of the bacilli. In addition to tests with acetone and water extracts of the bacilli, heat killed bacilli were also tested. The heat killed bacilli were prepared by taking a weighed quantity of actively growing bacilli and exposing them to flowing steam in an Arnold's Sterilizer for one hour each for two days. A suspension of these heat killed bacilli was prepared in different dilutions by means of sterile salt solution and added with aseptic precautions to tubes containing Long's medium. The medium was then melted, thoroughly mixed, slanted, and after cooling uniformly seeded with young actively growing tubercle bacilli. The results obtained with the heat killed bacilli in general were similar to those with the water extracts, in some cases a slight stimulating effect was noted while in most cases no effect on the growth of the bacilli was observed.

Since the result of the study of the effect of substances contained in tubercle bacilli on their growth proved to be irregular and indecisive, it was thought possible that the active principle, if such a substance existed, may have been destroyed by the heat used in the preparation of the substances tested. To eliminate the heat factor in the preparation of materials to be tested the tubercle bacilli were killed by placing them in a mixture of equal parts of absolute alcohol and ether for 48 hours at incubator temperature (37 C.) in a cotton plugged centrifuge tube. The ether-alcohol mixture was then evaporated off in vacuum, and a suspension prepared from the residue was placed on the surface of Long's medium. The growth of tubercle bacilli on this medium did not differ appreciably from that of controls, thus bearing out the preceding experiments and indicating an absence of growth-promoting principles in the tubercle bacilli themselves.

To determine whether the growth of tubercle bacilli is favored by products of their own metabolic activity or by products formed by their death and autolysis, an old tuberculin was prepared by growing virulent human (Gluckson) or virulent bovine strain of tubercle bacilli on Petroff's liquid medium. These preparations were found to give a typical tuberculin reaction in dilution as low as 1 : 1000. Different dilutions of these tuberculins were prepared and added to Long's medium. As a control the Petroff's liquid medium was evaporated down to the same concentration as the tuberculin preparation. Different dilutions of this concentrate were then also added to Long's medium, thus giving the same concentration of the nutrient salts in both the experimental and in control culture tubes. No acceleration of the growth of tubercle bacilli as a result of the addition of old tuberculin prepared from either virulent human or virulent bovine types of bacilli was noted. A slight increased growth of virulent bovine tubercle bacilli in some of the experimental series (10% tuberculin content) was most probably due to improvement of the medium for the growth of this strain of tubercle bacilli by the introduction of some organic substances (proteins?) and not due to any specific growth stimulant for tubercle bacilli contained in the tuberculin preparation. Usually the tubercle bacilli of the bovine strain do not grow well on Long's medium and an addition of some organic substance makes it a more favorable medium for the growth of the bacilli of this strain.

DISCUSSION

It is evident from this study that tubercle bacilli when seeded in suspension of 10 mg. or less per cc. are incapable of growing on Long's medium while suspensions of 50 mg. or more per cc. result in growth. Growth of tubercle bacilli can occur in mediums containing various complex substances occurring in nature even when in dilute suspensions. Moreover, when the growth rate of tubercle bacilli are compared among the heavily seeded cultures on Petroff's or Dorset's medium and those of Long's medium, the growth rate does not differ greatly among them which is not the case with lightly seeded cultures. This is in harmony with the view that some unidentified growth stimulant similar to the "bios" of Wildiers for the growth of yeast is essential to the growth of tubercle bacilli. The recent investigations of Burrows and Jorstad [19] indicate that even the growth of body cells is impossible

[19] Am. J. Physiol., 1926, 77, p. 24.

without the action of a substance or substances formed by them or by other cells in certain concentrations. All attempts to prove the presence of growth essential factors in the cells of tubercle bacilli or in their metabolic products have failed. The acetone extract, the water extract of the tubercle bacilli, or the dead bacilli themselves do not appear to have a stimulating effect on the growth of tubercle bacilli. The failure of these substances to stimulate growth is not due to the destruction of the active principle or principles by heat since bacilli killed by chemical means (alcohol and ether) at 37 C. did not enhance the growth of tubercle bacilli. Likewise, the metabolic products of tubercle bacilli prepared in the form of old tuberculin did not exert any favorable action on the growth of tubercle bacilli.

These results would not incline one to the belief that tubercle bacilli require growth essential factors such as are present in the bacillary cells themselves or in their metabolic products. If such substance or substances exist in the cells or in their metabolic products, proof of their presence requires much more delicate technic than that used in this study. The problem of "bios" and related subjects are still under dispute among scientific investigators and this problem as it concerns the tubercle bacillus requires further experimentation to demonstrate such principles if they exist.

SUMMARY

Tubercle bacilli fail to grow when seeded with suspensions containing 10 mg. or less of young active bacilli per cc., while a suspension containing 50 mg. or more per cc. grows in Long's synthetic medium containing $2\frac{1}{2}\%$ agar. On egg mediums growth occurs at lower concentration of suspensions of the bacilli.

All attempts to demonstrate the presence of a growth stimulating substance or substances in the cell of tubercle bacilli or in their metabolic products in the form of old tuberculin failed.

These results seem to indicate that the "bios" theory of Wildiers for yeast is not applicable for tubercle bacilli as an explanation of the failure of their growth when thinly seeded on artificial mediums.

FOOD ACCESSORY SUBSTANCES (VITAMINS) AND TUBERCLE BACILLI

2. THE EFFECT OF THE COMMONLY KNOWN VITAMINS ON THE GROWTH OF TUBERCLE BACILLI

NAO UYEI

From the Research Department, National Jewish Hospital, Denver, Colorado

The investigations by Lloyd,[1] Kligler,[2] McLeod and Wyon,[3] Thjötta and Avery,[4] and Davis [5] all indicate that water soluble B, or a vitamin of the similar nature is essential to the growth of pathogenic and non-pathogenic bacteria. As far as the writer has been able to learn no study of this nature has been made on tubercle bacilli with the exception of that by Long [6] who reported that there occurred no increased growth of tubercle bacilli when using the commercial yeast vitamins on the market. He did not, however, describe the method used nor give the data substantiating his findings. I found [7] that the growth of tubercle bacilli in no way appears to be dependent upon substances present within their cells or in their metabolic products and that mediums containing various organic substances were suitable for growth, while on suitable nonprotein synthetic mediums no growth occurred when the medium was seeded with a thin suspension of bacilli, indicating that the essential factor for the growth of the bacilli comes from a source other than from the bacilli themselves. The following study is concerned with the relation of the commonly known vitamins to the growth of tubercle bacilli.

Method.—Long's medium containing $2\frac{1}{2}\%$ agar was used as the basic medium and to this was added the substances in different amounts known to contain one or two different types of vitamins. The mediums were seeded with tubercle bacilli in suspensions containing 30 mg. of young actively growing bacilli per cc. The culture tubes were grown at 37.5 C. and the amount of growth was determined at weekly intervals. The vitamin accessory substances used in this study were the following:

"Oscodal" of Funk and Dubin[8] which is a cod liver oil concentrate and is known to contain fat soluble A and the antirachitic principle (vitamin D). (Material for these experiments was kindly furnished by Dr. Dubin).

[1] J. Path. & Bact., 1916, 21, p. 113.
[2] J. Exper. Med., 1919, 30, p. 31.
[3] J. Path. & Bact., 1921, 24, p. 205.
[4] J. Exper. Med., 1921, 34, p. 97; ibid., 1921, 34, p. 455.
[5] J. Infect. Dis., 1921, 29, p. 171.
[6] Am. Rev. Tuberc., 1922, 5, p. 705.
[7] J. Infect. Dis., 1927, 40, p. 425.
[8] Proc. Soc. Exper. Biol. & Med., 1923, 21, p. 139.

Harris' yeast vitamin (water soluble B).

Orange juice, tomato juice and cabbage juice, all of which are rich in water soluble B. and the antiscorbutic factor (vitamin C).

In preparing the culture medium for the experiments with "Oscodal," the vitamin-containing substance was dissolved in glycerol and different dilutions prepared using the same solvent, for introduction into the culture tubes containing Long's medium. The glycerol thus added, however, was found to exert a slight inhibitory effect on the growth of both human and bovine tubercle bacilli and consequently acetone was used as a substitute solvent for the "Oscodal" in place of the glycerol. One cc. of the acetone-solution of "Oscodal," was added to 9 cc. of Long's medium and the culture tubes thus prepared were heated at 96 C. for one hour each for two days in an Arnold sterilizer to drive off the acetone and sterilize the medium.

TABLE 1

THE EFFECT OF VITAMIN-CONTAINING SUBSTANCES ON THE GROWTH OF VIRULENT TUBERCLE BACILLI

Mediums	Dilution of Vitamin Containing Substance in the Medium	Human Bacilli (Gluckson)			Bovine Bacilli		
		1 Week	2 Weeks	3 Weeks	1 Week	2 Weeks	3 Weeks
Control.....................	0.00	±*	+	++	0	±	+
	0.00	±	+	++	±	±	+
Harris yeast vitamin.........	1:100	±	++	++++	+	+	++
	1:100	+	++	++++	+	+	++
Orange juice.................	1:10	+	+++	++++	±	++	++
	1:10	±	++	++++	±	++	++
Tomato juice................	1:10	++	+++	++++	+	+	++
	1:10	+	+++	++++	+	+	++
Expressed juice of cabbage..	1:10	±	++	+++	±	+	++
	1:10	±	++	++	±	+	++

* The growth of the culture is graded from no growth (0) to excellent (++++) growth.

For Harris' yeast vitamin, orange juice, tomato juice, and cabbage juice, distilled water was used in making the dilutions, and in every case 1 cc. of the solution was introduced into 9 cc. of Long's medium (table 1).

Results of experiments not recorded in the table indicate that the cod liver oil concentrate which is rich in fat-soluble A and the antirachitic principle exerted only an indifferent effect on the growth of tubercle bacilli, while all of the substances tested which are known to contain vitamin B or C in large amounts had a markedly favorable influence on the growth of the tubercle bacilli in vitro (table 1). In order to ascertain more definitely the nature of the growth stimulating principle present in the latter substances, tomato juice was concentrated to a small volume by vacuum distillation and a large amount of absolute alcohol added to make a final concentration of alcohol about 96%. The white precipitate resulting from this treatment was filtered off, freed

Fig. 1.—The stimulating effect of Harris' yeast vitamin and tomato juice on the growth of human tubercle bacilli. Tube 1 (Ca 2) shows a slight stimulating effect of Harris yeast vitamin in concentration of 1:100 over the growth obtained in the control, tube 3 (Ab. 3). Tube 2 (Fa 2) reveals a stimulating effect of fresh tomato juice 1 cc. added to 9 cc. of Long's medium. Tube 4 (Db 2) shows the marked stimulating effect of the alcoholic (96%) precipitate of tomato juice, and tube 5 (Ea 2) shows the stimulating effect of the alcoholic filtrate.

Fig. 2.—The stimulating effect of Harris' yeast vitamin and tomato juice on the growth of bovine tubercle bacilli. The tubes are arranged in the same order as in figure 1, and show similar results. The center tube 8 (Ab 5) is the control tube. Tube 6 (Ca 4) contained Harris yeast vitamin 1:100; tube 7 (Fa 6) contained fresh tomato juice 1 cc. in 9 cc. of Long's medium, tube 9 (Da 3) contained the 96% alcoholic precipitate of tomato juice and tube 10 (Ea 3) contained the alcoholic filtrate.

Fig. 3.—The stimulating effect of orange juice and cabbage juice on the growth of avirulent human tubercle bacilli. Tube 12 (6) is the control; tube 11 (a 3) contained 1 cc. of orange juice in 9 cc. of Long's medium while tube 13 (a 4) contained 1 cc. of cabbage juice in 9 cc. of Long's medium.

from alcohol by vacuum distillation, dissolved in water and was then added to Long's medium in different concentrations. The alcoholic filtrate was also freed from alcohol by vacuum distillation, was dissolved in water and added to Long's medium in different concentrations.

The results of the culture experiments indicate that the active principle contained in tomato juice can be precipitated out by means of strong (96%) alcohol, although a large amount of the active substance must still have remained in the filtrate. In view of the fact that Osborne and Wakeman [9] were able to precipitate out the water soluble vitamin of Brewer's yeast by means of 79% alcohol, their method of fractional precipitation was applied to tomato juice in order to study the solubility behavior of the growth stimulating substance in this way for tubercle bacilli. Concentrated tomato juice was successively treated with 52%, 79% and 90% alcohol.

The precipitates formed were filtered off, dissolved in water, and after vacuum distilling off of the alcohol, were added in different concentrations to Long's medium which was then seeded with tubercle bacilli. Contrary to expectation the growth stimulating substance contained in the tomato juice remained mostly in the filtrate from 90% alcohol precipitate, although some of it was precipitated, indicating that the growth stimulating substance is soluble in dilute alcohol, but sparingly soluble in concentrated alcoholic solutions. This solubility behavior toward alcohol is characteristic for the water soluble vitamin contained in many of the substances tested by other investigators.

DISCUSSION

The fact that the fat soluble A and the antirachitic principle contained in the cod liver oil concentrate (Oscodal) is indifferent or rather has a slightly inhibitory effect on the growth of tubercle bacilli is in harmony with the findings by Manville [10] and others for other bacteria. In contrast to this lack of stimulating action of fat soluble A and D on the growth of tubercle bacilli are the substances which are known to contain a large amount of water soluble B or C which exert a pronounced stimulating effect on the growth of tubercle bacilli and this stimulating effect is observed with concentrations of 1 : 1000 for the Harris yeast vitamin, 1 : 100 for the orange juice and tomato juice and 1 : 20 for the cabbage juice.

The question naturally arises whether the stimulating action on the growth of the tubercle bacilli is due to vitamin B or C. Since there is no

[9] J. Biol. Chem., 1919, 40, p. 383.
[10] J. Cancer Res., 1925, 9, p. 254.

evidence at present available to indicate that the antiscorbutic factor (vitamin C) is necessary for the growth of lower organisms, it does not appear reasonable that the activity of the substances tested on the growth of tubercle bacilli is due to the presence of vitamin C. The investigations of Thjötta and Avery, Davis, Kligler and others, appear to definitely indicate that vitamin B or a substance of similar nature is essential for the growth of bacteria. The experiments recorded in this study demonstrate that the growth stimulating substance contained in the tomato juice is readily soluble in dilute alcohol (79% or below) but sparingly so in concentrated alcohol (90% or above), indicating a solubility behavior like the water soluble vitamin contained in most substances. The dissimilarity in solubility between the active principle of tomato juice and the water soluble vitamin of Brewer's yeast cannot be considered as evidence against a consideration of the former as water soluble B, since the solubility of the water soluble B may differ according to the conditions under which they are found.[11] Since the true chemical nature of vitamin B has not yet been established, it is impossible to state whether the growth stimulating substance present in Harris' yeast vitamin, orange juice, tomato juice and cabbage, is vitamin B or not. But since all of these substances contain vitamin B and the solubility behavior of the active principle in the tomato juice resembles that of vitamin B present in most substances and the necessity of vitamin B for the growth of other bacteria has been demonstrated by others, it is believed that the active stimulating substances essential for the growth of bacteria in Harris' yeast vitamin, orange juice, tomato juice and cabbage juice is vitamin B or a vitamin of similar nature, and that such vitamin or vitamins are essential to the normal growth of tubercle bacilli.

SUMMARY

Cod liver oil concentrate ("Oscodal" of Funk and Dubin) known to be rich in fat soluble A vitamin and the antirachitic vitamin exerts an indifferent action on the growth of tubercle bacilli.

Conforming with the results of other investigators with other bacteria, substances rich in vitamin B, namely, Harris' yeast vitamin, orange juice, tomato juice and cabbage juice exert a pronounced stimulating effect on the growth of tubercle bacilli. With the technic used in these studies, the acceleration of growth is easily discernible in concentration of 1 : 1,000 for Harris' yeast vitamin, 1 :1,00 for tomato juice and orange juice and 1 : 20 for the cabbage juice.

[11] Ellis and McLeod: Vital Factors of Food, 1922, p. 115.

THE NECESSITY OF CARBON DIOXIDE FOR THE GROWTH OF BACTERIA, YEASTS AND MOLDS

GEORGE E. ROCKWELL AND JOHN H. HIGHBERGER

From the Department of Bacteriology and Hygiene, University of Cincinnati

The subject of bacterial metabolism is at present in a vague and unsatisfactory state. We know that carbon is the structural unit of living material, and that bacteria, in common with all living things, require, in addition to this element, nitrogen, oxygen, hydrogen and inorganic salts. It is usually stated that a certain degradation of organic material takes place, and that the products are absorbed into the bacterial cell, where they are resynthesized into the complex substances which compose protoplasm. Just how far this degradation proceeds before absorption takes place, and in what form the structural materials are taken into the cell, has never been clearly defined.

The writers have felt for a long time that carbon dioxide plays an essential role in bacterial metabolism. So far as we are aware, the first suggestion in the literature that this gas is directly concerned in the growth of a pathogenic organism was made by Wherry and Ervin,[1] who showed that its presence in a certain minimum tension was necessary for the cultivation of the tubercle bacillus.

In 1921 one of us showed that carbon dioxide favors the growth of certain aerobes and facultative anaerobes,[2] and, somewhat later, that the presence of the gas is necessary for anaerobic growth.[3] The subject has been continuously under investigation in this laboratory since the date mentioned above, and, although during the whole course of the work we have been under the strong conviction that carbon dioxide is an essential to the growth of all organisms, it has never been felt that the experimental evidence was extensive enough to warrant the claim of absolute proof. Furthermore, several authors have objected to considering carbon dioxide as directly concerned in bacterial growth, and have ascribed the favoring influence of added carbon dioxide to other indirect factors, such as the alteration of the reaction of the medium,[4] better retention of moisture,[5] or reduced oxygen tension.[6] Others have claimed that it has no influence whatever. Novy and Soule,[7] for example,

[1] J. Infect. Dis., 1918, 22, p. 194.

[2] Ibid., 1921, 28, p. 352.

[3] Ibid., 1923, 32, p. 98; 1924, 35, p. 581.

[4] St. John, J. H.: Med. Rec., 1919, 95, p. 184. Kohman, E. F.: J. Bact., 1919, 4, p. 571. Gates, F. L.: J. Exper. Med., 1919, 29, p. 321.

[5] Erickson, M. J., and Albert, H.: J. Infect. Dis., 1922, 30, p. 268. Torrey, J. C., and Buckell, G. T.: ibid., 1922, 31, p. 125.

[6] Herrold, R. D.: J. Am. M. A., 1920, 74, p. 1716. Swartz, E. O.: J. Urol., 1920, 4, p. 325.

[7] J. Infect. Dis., 1925, 36, p. 168.

were unable to confirm the results of Wherry and Ervin. Recently, however, we have demonstrated that carbon dioxide is undoubtedly a factor in the growth of the tubercle bacillus,[8] and have pointed out certain factors, lack of control of which probably explain Novy's results. The evidence was also extended to other acidfast organisms.

The idea that carbon dioxide is concerned in the growth of micro-organisms is evidently now beginning to find more favor. Confirmatory evidence is given by the work of Theobald Smith[9] on B. abortus, as well as that of Valley and Rettger,[10] who have recently added several organisms to the list of those which have been demonstrated to be inhibited in the absence of the gas. We feel that the experiments detailed in the present paper, taken in conjunction with previously reported work on facultative and strict anaerobes, and on the group of acidfast organisms, justify us in the conclusion that carbon dioxide is involved directly in the growth of all bacteria, yeasts and molds.

EXPERIMENTAL

Since the simplest available method of investigating the carbon dioxide relations of an organism consists in determining if growth is possible or inhibited in the absence of the gas, we have followed this plan in the present work. The methods and general technic, which have

TABLE 1

GROWTH OF ORGANISMS ON MEAT EXTRACT AGAR, REACTION +0.5, INCUBATED 24 HOURS AT 37 C.

	B. Coli	B. Typhosus	B. Proteus	B. Pyo-cyaneous	Staphylococ-cus Albus
Control, aerobic................	+++	+++	+++	+++	+++
Incubated over 20 cc. 4% NaOH..	+++	±	+++	+++	0

been described in a previous publication,[8] consist, briefly, of the incubation of plate cultures over alkaline solutions in sealed desiccators, with suitable controls for oxygen volume, dehydration of the medium, etc.

Table 1 shows the growths of five different organisms on meat extract agar plates incubated over an alkaline solution, and also aerobically for controls.

It is seen that under these conditions only two of these organisms, namely: B. typhosus and staphylococcus albus, show inhibition of growth over the alkaline solution. We have previously pointed out that negative results, by which we mean failure to obtain inhibition over the alkali while there is normal growth on the corresponding control, cannot be considered conclusive unless due attention is given to four factors, which were discussed at that time. (A fifth factor has since been found and is discussed later on.) Briefly, these are: the presence of carbohydrates in

[8] J. Infect. Dis., 1926, 38, p. 92.
[9] J. Exper. Med., 1924, 40, p. 219.
[10] Abstr. Bact., 1925, 9, 1771; J. Bact., 1926, 11, p. 78.

the medium; the protein content of the medium; the amount of the inoculum; the reaction of the medium, and the concentration of salt in the medium (shown in this paper to be a fifth factor).

An opportunity is here accorded us of demonstrating the applicability of these points. It is to be noted that the organisms which gave negative results in the above experiment are those which produce carbon dioxide most readily from the proteins of the medium. By reducing the protein content of the medium to the point which just sustains growth on the aerobic control, we are able to demonstrate inhibition of growth over the

TABLE 2

Growth of B. Coli on Various Dilutions of Meat Extract Agar with Agar-Water

	Percentage of Meat Extract Agar			
	100	50	25	12.5
Aerobic control..................................	+++++	++++	+++	++
Incubated over 20 cc. 4% NaOH...............	+++++	++++	++	±

TABLE 3

Growth of Staphylococcus on Meat Extract Agar of Reaction +0.5, When the Amount of Inoculum Is Varied

	Incubated 18 Hours at 37 C.			
	Inoculated Directly from 24 Hour Slant	Dilutions		
		1	2	3
Aerobic control...............................	+++++	+++	++	+
Incubated over 20 cc. 4% NaOH..............	++++	++	+	0

TABLE 4

Growth of B. Coli on Meat Extract Agar, Initial Reaction +0.5

	Cc. of N/5 Lactic Acid Added to 10 Cc. of Medium					
	0	0.4	0.5	0.6	0.7	0.8
Aerobic control..........................	++++	++++	++++	++++	+++	+++
Incubated over 20 cc. 4% NaOH............	++++	+++	++	±	0	0
Incubated over 20 cc. 10% H2SO4..........	++++	+++	+++	+++	+++	+++

alkaline solution. This is shown for B. coli in table 2, where the reduction of the protein content was secured by diluting a regular meat extract agar of reaction + 0.5 with various proportions of a jell composed only of agar and water.

In order to obtain clean cut evidence of growth inhibition, the inoculum must be reduced to the minimum, and spread uniformly over the medium. This is shown for the staphylococcus in table 3.

In Table 4 is shown the effect of modifying the reaction of the medium.

It is apparent from the data in table 4 that when the medium is made sufficiently· acid, inhibition of growth over the alkaline solution becomes evident. The effect of acidifying the medium in this way is a triple one: it cuts down proteolytic action, which means decreased production of carbon dioxide; it renders the carbon dioxide more available to the organisms; and it facilitates the removal of the gas from the medium by the alkaline absorbent. Similarly in table 5, the necessity of carbon dioxide for the growth of a mold is demonstrated, with the effect of changes in the reaction of the medium.

TABLE 5

Growth of Mucor on Meat Extract Agar, 18 Hours at 37C.

| | Cc. of N/1 Lactic Acid Added to 10 Cc. of Medium | | | |
	1.8	1.9	2.0	2.1
Aerobic control..........................	++++	++++	+++	++
Incubated over 20 cc. 4% NaOH...............	++	++	+	0
Incubated over 20 cc. 30% glycerol............	++++	++++	+++	++

TABLE 6

Growth of B. Pyocyaneous on Meat Extract Agar of Initial Reaction +0.5, 24 Hours at 37 C.

| | Cc. of 1.027 N/5 Lactic Acid Added to 10 Cc. of Medium | | | | | |
| | 0.69 | | 0.70 | | 0.71 | |
	Growth	% Dehydra- tion	Growth	% Dehydra- tion	Growth	% Dehydra- tion
Aerobic...........................	+++	6.2	++	5.9	++	7.3
Incubated over 20 cc. 30% glycerol.......	+++	19.8	++	15.5	++	16.1
Incubated over 20 cc. 4% NaOH..........	+++	7.8	+	4.9	±	7.2

Inhibition of growth of B. pyocyaneous over alkali on an acid medium is shown in table 6. The figures are also given which indicate that the growth inhibition cannot be ascribed to dehydration of the medium.

With such markedly proteolytic organisms as the strain of B. pyocyaneous used in the above experiment the reaction adjustment necessary to demonstrate inhibition over alkali is a delicate one, due to the great sensitivity of the organisms to acid. It is hardly necessary to observe that the exact reaction, expressed in the amount of acid added to the medium, at which growth inhibition over alkali occurs, varies with each lot of medium.

During the later course of this work a fifth factor, in addition to the four already discussed, was found to influence the results of the experiments. It was discovered that with the presence of certain concentrations of sodium chloride in mediums of normal acidity, growth inhibition over alkaline solutions cannot be demonstrated. This is shown for the staphylococcus in table 7. That this does not mean that under these conditions carbon dioxide is not necessary for growth, is shown in table 8. By modifying the reaction of the medium, a point of acidity is

TABLE 7

STAPHYLOCOCCUS ON MEAT EXTRACT AGAR, REACTION +0.5. INCUBATED 20 HOURS AT 37 C.

	% NaCl in Medium				
	Control 0.5	3	5	8	Control 0.5
Aerobic control..................	++++	++++	+++	++	++++
Incubated over 20 cc. 4% NaOH..	∓	++	+++	++	∓

TABLE 8

STAPHYLOCOCCUS ON DILUTED MEAT EXTRACT AGAR INITIAL REACTION +0.5. INCUBATED 38 HOURS AT 37 C.

	Cc. N/50 Lactic Acid Added to 10 Cc. Medium					
	0	0	0.01	0.025	0.05	0.075
% NaCl.....................................	½	2	2	2	2	2
Aerobic control............................	++++	++++	++++	++++	++++	+++
Incubated over 4% NaOH..................	0	++	· +	±	∓	0
Incubated over 20 cc. 40% glycerol.........	++++	++++	++++	++++	++++	+++

TABLE 9

GROWTH OF SACCHAROMYCES ON ½ STRENGTH MEAT EXTRACT AGAR, 0% NaCl, 0.2% DEXTROSE, 65 HOURS AT 37 C.

	Colonies Developed
Aerobic...	2,000
Incubated over 20% glycerol..	3,000
Incubated over 20 cc. 4% NaOH..	1

finally reached where growth inhibition over alkali is evident, even in the presence of the sodium chloride. This experiement is illustrated in figure 1. The concentration of salt in the medium thus becomes the fifth factor which must be controlled in demonstrating growth inhibition over alkalies. For some organisms it is not so important; for others, as for example the strain of yeast with which we worked, it was found necessary to exclude even the ordinary concentrations of salt from the
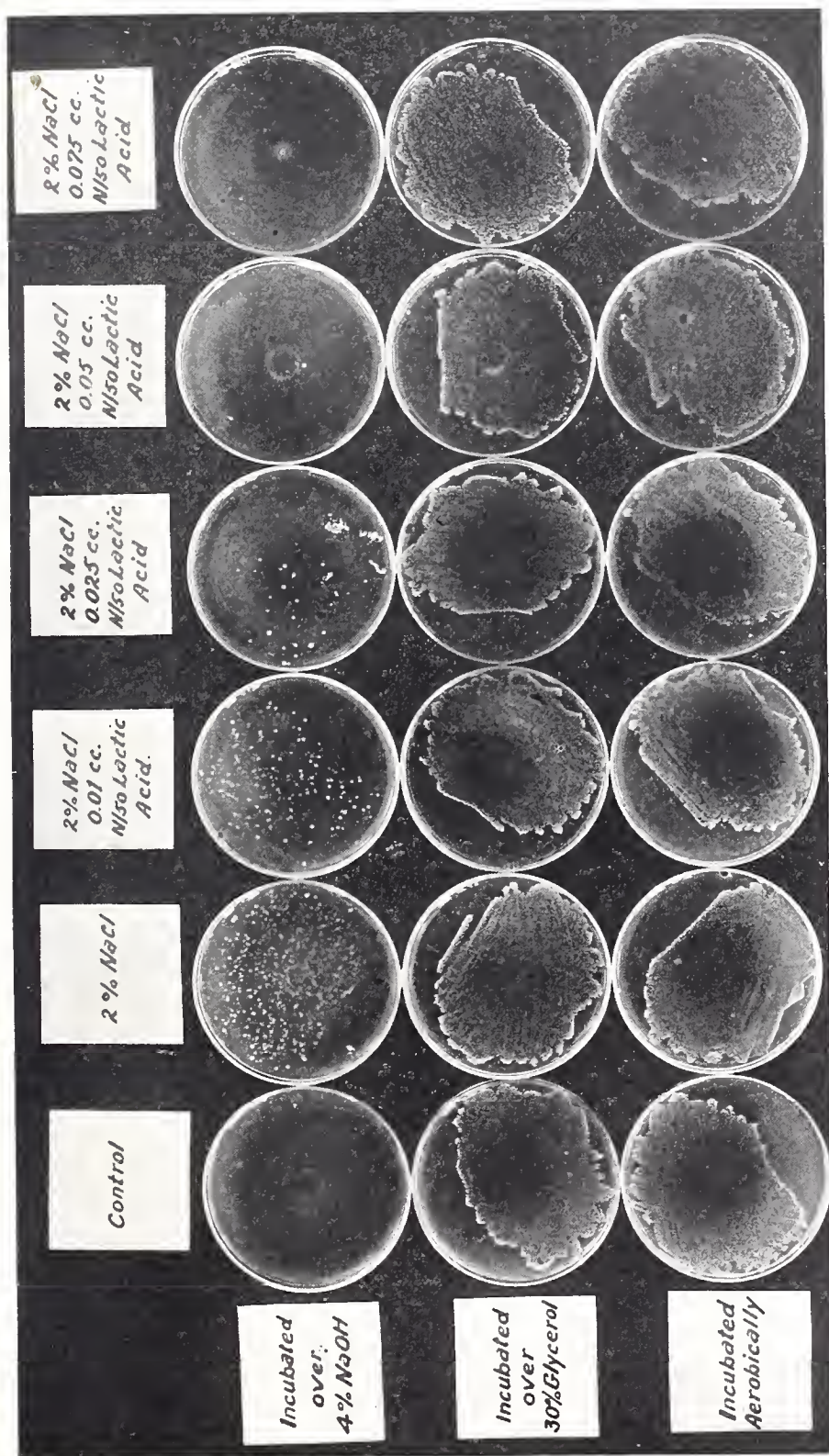
Fig. 1.—The inhibition of Staphylococcus albus over alkali, prevented by sodium chloride in the medium, and the disappearance of this effect with increasing acidity.

medium. This phenomenon has been made the subject of a separate study, the results of which will be reported in a later publication. In table 9 is shown the application of the above discussed factors in demonstrating the necessity of carbon dioxide for the growth of a yeast.

The yeast, for which results are given in table 9, was a saccharomyces used in industrial alcoholic fermentation, and was consequently a copious producer of carbon dioxide, which rendered the demonstration of growth inhibition over alkali very difficult. On the other hand, results could probably be obtained more easily with other species of yeasts which have less marked fermentative powers, such as some species of torula, and certain pathogenic yeasts.

DISCUSSION

It is shown in this paper that when the proper conditions are provided, growth inhibition over alkali can be demonstrated for several species of bacteria, yeast and molds. Inhibition of growth over alkaline solutions cannot be interpreted in any other way than as the effect of the removal of carbon dioxide, as is shown by the following considerations:

The inhibition of growth is not due to dehydration of the medium by the alkali, as it has been shown previously, as well as in the present experiments, that growth takes place under conditions of much greater dehydration than that induced by the alkaline solutions employed.

The inhibition of growth is not due to any effect of the alkali upon the reaction of the medium. Novy has shown that no change in reaction occurs under these conditions, and our own experiments have also indicated this.

The inhibition of growth is not due to any reaction change induced in the medium by the removal of carbon dioxide. Such a change could only be to the alkaline side, and it is shown above that making the medium more acid facilitates the demonstration of growth inhibition over alkali.

All these facts, taken in conjunction with certain cultural characteristics which are constantly being brought under observation, such as the striking results obtained with the carbon dioxide charged solution for anaerobic culture previously described by one of us, have led us to the conclusion that all microorganisms of these classes require carbon dioxide for growth. It is, perhaps, in the present state of our knowledge, a little premature to speculate on the probable way in which carbon dioxide influences growth. We can only point out that there are several possibilities, among which are: utilization as a source of carbon; stimulation of reproduction; necessary stimulus to the mechanism controlling respiration; activation of the bacterial enzymes.

At the present time it is our opinion that carbon dioxide is utilized by bacteria as a source of carbon, and that it is the only source which can be so utilized. The work of Winogradsky, Beijerinck, Nathanson, and others, has demonstrated beyond a doubt that certain species of bacteria do obtain their carbon from this source. In our belief, these are not merely isolated instances, but are in reality examples of a general phenomenon, made more easily demonstrable by the peculiar nature and activities of the organisms in question. We are instituting in this laboratory a quantitative study of the carbon metabolism of bacteria, the results of which will be reported in a future publication.

Due to the fact that the influence of carbon dioxide on growth was first noticed in connection with pathogenic organisms on primary cultivation from the body, the possibility of a relation between pathogenicity and response to growth stimulation by carbon dioxide has been suggested. We do not believe that any such relation exists, although there is no doubt that the favoring influence of added carbon dioxide on growth is best seen in the case of infecting organisms freshly isolated from the host. We believe that the parasite, adapted to the native proteins of the tissues of its host, obtains its carbon dioxide with difficulty from the more or less denatured proteins of the artificial medium, and thus responds to the artificially added gas to a greater degree than saprophytic organisms, which can obtain their carbon dioxide from the medium with more or less ease. The acquisition of a degree of saprophytism by a parasite simply means, in our opinion, among other factors, the gradual development of the ability to produce from the artificial medium sufficient carbon dioxide for its needs.

SUMMARY

Growth inhibition of several strains of bacteria, mold, and yeast, incubated over an alkaline solution is shown. Factors which govern the demonstration of growth inhibition are: the presence of carbohydrates in the medium, the protein content of the medium, the amount of the inoculum, the reaction of the medium, and the conentration of salt in the medium.

The opinion is expressed that all bacteria, yeasts and molds require carbon dioxide for their growth, and that the gas is used as a source of carbon.

Authors' Note: Since this paper was accepted for publication a paper by Masur,[11] has come to the attention of the authors. He states that he has cul-

[11] Masur, B. L.: Centralbl. f. Bakteriol. 1, o., 1926, 99, p. 46.

tivated a strain of tubercle bacilli on a carbon free medium for sixteen genera-
tions, and he concludes that "under certain conditions the tubercle bacillus can
utilize directly the carbon of the carbon dioxide of the air." He considers
the ammonia in his synthetic medium to be the source of the necessary energy.
An experiment is cited showing that the culture will not develop in the
absence of the carbon dioxide of the air. No reference is made to any other
work on the same subject, and Masur is apparently not aware that any has
been done.

METAPHEN (4-NITRO-3,5-BISACETOXYMERCURI-2-CRESOL) AND ITS BACTERICIDAL PROPERTIES

GEORGE W. RAIZISS AND M. SEVERAC

From the Dermatological Research Laboratories, Philadelphia

Mercury compounds have long been known as bactericidal agents. Schamberg, Kolmer and Raiziss [1] in a paper on inorganic and organic mercury compounds stressed their highly destructive effect on bacteria, and in later papers [2] called attention to the highly parasiticidal effect of a mercury derivative, sodium oxy-mercury-ortho-nitro-phenolate, which they named mercurophen. The authors synthesized this mercury compound in the belief that the presence of the nitro group in the molecule was particularly desirable in conjunction with the nuclear mercury for the purpose of stabilizing the molecule, and also in order to obtain a maximum destructive effect on bacteria by building up a nitro-benzene-mercury complex. In subsequent years, the present authors further developed this idea by investigating many chemical compounds carrying the above mentioned nitro-benzene-mercury complex. Numerous substances were studied for toxicity and bactericidal properties. As a result, the authors were much impressed with the germicidal effect of a substance they called metaphen. Chemically it is 4-nitro-3,5-bisacetoxymercuri-2-cresol. Laboratory experiments disclosed its superiority over many other germicidal substances. One of the most powerful of these is bichloride of mercury, and a chemical compound which approaches the power of bichloride of mercury is worthy of attention. For many strains of bacteria metaphen is much more powerful than bichloride of mercury, sometimes to an astounding degree. The specific effect on bacteria is greatly increased when bacteria remain in contact with metaphen for more than one hour. This rapidly mounting destructive effect is not observed in the same degree with other germicidal substances including bichloride of mercury. The inhibiting effect of metaphen upon the growth of staphylococcus aureus [3] within the first 24 hours is noticeable in such large dilutions as 1 : 20,400,000. The above figure is an average for six experiments. The bactericidal effect

[1] Am. J. Syph., 1917, 1, p. 1.
[2] J. Am. M. A., 1917, 68, p. 1458; J. Infect. Dis., 1919, 24, p. 547.
[3] Raiziss, G., and Severac, M.: J. Lab. & Clin. Med., 1923, 9, p. 71.

which expresses the actual killing power of the chemical compound is 1 : 3,850,000 after four days' contact of the bacteria with metaphen. In this particular experiment the bichloride of mercury index was 11.46 and the germicidal index 45.74.* The high bactericidal effect of metaphen on the group of cocci is further shown in its bactericidal values for the streptococci. Six tests (average in table 1) were performed on Streptococcus hemolyticus, according to the following technic.

Exposure of Bacteria to the Chemical for 1 Hour.—This was carried out as follows: Solutions of the chemicals in sterile water were made up in varied concentrations, and 4 cc. of each dilution were placed in each of a series of sterile test tubes. The test organisms used were grown in broth for 24 hours, shaken with beads to break up clumps, diluted with sterile physiologic salt solution until a definite suspension was obtained by comparing it with the standard adopted in our laboratories, and 1 cc. added to each test tube making the total volume 5 cc. After

TABLE 1

Bactericidal Action of Metaphen Compared with Other Germicides

	Dilutions Bactericidal for Organisms Exposed for Indicated Time Periods								
	Streptococcus Hemolyticus		Gonococcus		Pneumo-coccus	B. Anthracis		B. Subtilis	
	(6)* 1 Hour	(5)* 4 Days	(6)* 15 Min.	(6)* 30 Min.	(4)* 1 Hour	(6)* 1 Hour	(6)* 4 Days	(3)* 1 Hour	(7)* 4 Days
Metaphen............	90,000	3,140,000	380,000	380,000	55,000	326,000	8,000,000	133,000	2,640,000
Bichloride of mercury	125,000	180,000	....	....	42,000	29,000	170,000	25,000	103,000
Mercurochrome.......	5,300	24,000	56,300	66,300	2,500	530	31,000	230	17,000
Phenol...............	200	200	333	400	50	133	1,860	100—	290

* Average of indicated ( ) number of tests.

the culture had been added to the solution of the chemical, the test tubes were incubated for one hour at 37 C., and subcultures made. These subcultures were observed at the end of 24 and 48 hours.

Exposure for 4 Days.—In this series of experiments only 1 cc. of each dilution of the chemical was placed in each test tube. The test organism was grown in broth for 24 hours, shaken with beads to break up the clumps, and 1 cc. added to 100 cc. of broth of which 5 cc. were added to each test tube, making the total volume 6 cc. The test tubes were then incubated for four days at 37 C., and observed every 24 hours. The tubes were considered sterile when they looked perfectly clear, but to determine whether complete destruction of all the bacteria took place, subcultures were made at the expiration of the incubation period. In each experiment controls of the cultures and sterile salt solution were included.

A glance at this table discloses the superiority of metaphen over phenol and mercurochrome in exposure for one hour as well as in four days exposure. Again, it has been observed that a longer contact of

* The bichloride of mercury index is obtained by considering the germicidal power of mercuric chloride as unity, and computing the corresponding values of the other chemicals. The germicidal index is the product obtained by multiplying the bichloride of mercury index by the maximum tolerated dose for white rats (in mg. per kilo of body weight).

metaphen with bacteria greatly increases its bactericidal effect, a thing which does not occur to such an extent with bichloride of mercury or phenol. In a four days exposure metaphen destroys Streptococcus hemolyticus in such large dilutions as 1 : 3,140,000. The powerful effect of metaphen on the gonococcus and the pneumococcus is also demonstrated. These figures disclose the superiority of metaphen over bichloride of mercury, mercurochrome and phenol, metaphen being 22 times more powerful than mercurochrome and 1,100 times more powerful than phenol.

Another striking feature which makes metaphen a good general bactericidal substance is its powerful destructive effect not only on various representatives of the group cocci but also on many other bacteria. This quality of metaphen puts it in the class of pantherapeutic agents. While being uniformly very high in its bactericidal properties, its effect on some bacteria is specifically high. Our research on the effect of metaphen on the group of spore bearing bacilli disclosed amazingly high figures for the anthrax. Here the superiority of metaphen over bichloride of mercury reaches its highest point, as shown in the table. In a one hour exposure metaphen is thus 11 times more powerful than mercuric chloride and 615 times more powerful than mercurochrome. In a four days' exposure metaphen kills the anthrax bacillus in a dilution of 1 : 8,000,000, i. e. it is 47 times more powerful than bichloride of mercury, 258 times more powerful than mercurochrome, and 4300 more powerful than phenol. In the figures showing the effects on another spore bearer, Bacillus subtilis, the superiority of metaphen over bichloride of mercury, mercurochrome and phenol is very striking. Other experiments performed in our laboratories demonstrate the significant superiority of metaphen over bichloride of mercury and other chemical compounds on diptheria bacilli, Bacillus pyocyaneus and Bacillus coli. Space does not permit to go into detail here. The results of these experiments will be published elsewhere. The findings of Peterson[4] should be mentioned. After investigating the inhibitory effect of a great many mercury compounds on the yeast-sugar fermentation, he placed metaphen in the first class among the most efficient compounds he had used, saying "that the powerful inhibitory effect of metaphen is due to at least two factors; the effect of the mercury and the effect of the rest of the molecule."

Before accepting a germicidal substance in the treatment of disease, its toxicity, its combining power with serum proteins, its effect on tis-

[4] J. Am. M. A., 1926, 87, p. 223.

sues, and many other properties must be carefully considered. The intravenous administration of metaphen in animals shows that it is generally less toxic than bichloride of mercury. It is somewhat more toxic than the other chemical compounds discussed in this paper, but considering its powerful destructive effect on bacteria and comparatively low affinity for serum proteins its advantages over other chemical compounds even for intravenous use are quite obvious. However, metaphen finds its main application in the sterilization of infected mucous membranes, and since its advantage lies in surface sterilization, its toxicity

TABLE 2

Toxicity of Metaphen and Other Germicides as Indicated by Survival of Rats for 10 Days or More (S) After Intravenous or Intramuscular Injections

Compound	Dose, Gm. per Kg.	Results of Injections	
		Intravenous	Intramuscular
Phenol..	0.100	—	S
	0.300	—	S
	0.500	—	Death, 24 hours
Mercurochrome..............................	0.032	S	—
	0.036	Death, 4th day	—
	0.050	—	S
	0.100	—	S
	0.200	—	Death, 4th day
Mercuric chloride............................	0.002	S	—
	0.003	Death, 5th day	—
	0.006	—	S
	0.008	—	S
	0.010	—	Death, 5th day
Metaphen......................................	0.006	S	—
	0.007	Death, 7th day	—
	0.010	—	S
	0.020	—	S
	0.030	—	Death, 4th day

The dash (—) indicates no test made.

by the intramuscular route should be the chief consideration. Table 2 shows the toxicity of metaphen and other compounds given intravenously and intramuscularly. Metaphen appears to be about one-fourth as toxic intramuscularly as intravenously. It is distinctly less toxic than mercuric chloride.

Further evidence of the low toxicity and nonirritating effect of metaphen came to our attention in the oral administration of the drug to rabbits. We gave large quantities of metaphen by way of the stomach tube with little or no harmful effect. Rabbits can stand 30 to 40 mgs. per kg. of body weight. A rabbit weighing about 2 kg. can thus tolerate more than 60 cc. of a 1 : 1,000 solution of metaphen. We performed a considerable number of such experiments and are now convinced that absorption of metaphen by wounds, or swallowing it in small quantities

will not injure the patient. For example, one rabbit previously inoculated with Treponema pallidum received 46 treatments consisting of daily administrations of 2 cc. metaphen 1 : 1,000 without manifestations of toxicity and without any reactions. The spirochetes disappeared from the lesions after seven days' treatment, and the rabbit remained normal for 83 days.[5]

Metaphen was given the most extensive use in experimental surgical work in our laboratories. We performed numerous operations using nothing but metaphen as an aseptic precaution. Illustrative of the high bactericidal properties of the drug is its effect on the eyes of a physician when much gonococcic pus splashed into them during an experiment. One immediate application of a 1 : 1,000 solution saved the eyes from infection. On another occasion, fluids containing motile spirochetes were splashed into the eyes of the experimentor. An immediate application of metaphen prevented a syphilitic infection. The bacterial effect of metaphen in vivo was also demonstrated in the following laboratory experiment. Eyes of rabbits were infected, according to the method described by Ben Witt Key,[6] by deeply inoculating the cornea with a virulent strain of staphylococcus aureus. Control animals developed a strong infection, resulting in the appearance of corneal abscesses within 24 hours. Animals treated with metaphen two hours after inoculation by instilling into the eye two drops of a 1 : 1,000 solution and being similarly treated for the next 9 days, remained free from infection. The treatment was then stopped. The animals remained normal for the next ten days. Since metaphen has been applied only to the surface of the cornea and in comparatively small amounts, the experiment demonstrates that metaphen possesses considerable penetrative and curative properties.

The strong bactericidal effect of metaphen has also been demonstrated in a very wide field of clinical application. Metaphen appears to be particularly useful in the treatment and prevention of common cold. Sullivan[7] and Brown[8] have reported favorable results with the use of metaphen in the treatment and prevention of the common cold. Fenton[9] also states that in dilutions 1 : 5,000 to 1 : 10,000 metaphen seems to be a most useful organic mercury compound for otorhinologists; it is without objectionable local or renal irritation when used in sinus

[5] Raiziss and Severac: Progress in Chemotherapy, 1925, p. 21.
[6] J. Am. M. A., 1924, 82, p. 183.
[7] Sullivan: Clin. Med., 1925, 32, p. 150.
[8] Brown: Ibid., p. 181.
[9] Fenton: Ann. Otol., Rhinol. & Laryngol., 1925, 34, p. 801.

lavage, and is about equally bactericidal for staphylococci and strepto-
cocci. Finally, Kolmer,[10] in his researches on the chemotherapy of
gingivitis, found metaphen vastly superior to any of the other bacteri-
cides used. He says that although he has "not worked with this com-
pound (metaphen) as extensively as with mercurophen—its (i. e.
metaphen's) bactericidal activity is undoubtedly somewhat higher and
worthy of great interest from the standpoint of the treatment of gingi-
vitis by local applications."

SUMMARY

Metaphen possesses unusually high bactericidal properties, especially
when left in contact with the microorganisms for more than one hour,
in which respect it is more effective than any of the other germicides we
have employed. It is almost four times less toxic when injected intra-
muscularly than when injected intravenously. The toxicity when given
by mouth is also comparatively low. It is nonirritating in therapeutic
dilutions. Experimental work on animals as well as clinical observations
confirm its high efficiency as a bactericide.

[10] Kolmer: Dental Cosmos, 1926, 68, p. 1.

THE PURIFICATION OF ABORTIN

P. Schoenholz and K. F. Meyer

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

The studies of Fleischner and Meyer [1] have shown that guinea-pigs infected with Brucella abortus develop cutaneous hypersensitiveness which may be detected by the intradermal inoculation of suspensions of dried Br. abortus cultures or of broth autolysates. Difficulties were repeatedly encountered [2] in the preparation of abortins, typhoidins, etc., nontoxic to normal skins but effective in eliciting cutaneous reactions in infected animals. An attempt was therefore made to purify the abortin. In this connection the principles set forth by Zinsser and his co-workers and by Long and Seibert in the purification of tuberculin have been investigated. From the beginning the data collected in this laboratory did not corroborate the findings on tuberculin published in the earlier papers by Zinsser and his co-workers. Since the more recent reports from the same laboratory have refuted the deductions drawn from the preliminary work, it is deemed unnecessary to review in detail the earlier paper.

For the past few years Zinsser has maintained that the active principle which elicits the skin reaction in the tuberculous guinea-pig is not of protein nature. In collaboration with Parker [3] he noted that both the "nucleoprotein" and the "residue antigen" gave skin reactions, the former always stronger than the latter. These two workers reasoned that the skin reactive substance in the residue was derived from the nucleoprotein since the residue antigens reacted strongly and specifically with immune serums in vitro. In a later paper Zinsser [4] states "the active principle of tuberculin which elicits reactions in the sensitive subject is not a protein." It is only since the recent work of his collaborator Mueller [5] that the skin reactive antigens and the precipitating antigens have been separated. This investigator concludes that the cause of the skin reaction is probably but not surely a protein. He has furthermore been able to isolate a nonprotein gum from tuberculins pre-

[1] Am. J. Dis. Child., 1918, 16, p. 268.
[2] J. Infect. Dis., 1917, 20, pp. 357, 391.
[3] J. Exper. Med., 1923, 37, p. 275.
[4] Proc. Soc. Exper. Biol. and Med., 1924, 22, p. 35.
[5] Ibid., p. 209; J. Exper. Med., 1926, 43, pp. 1, 9.

pared on synthetic mediums which precipitates in high dilutions in the presence of the homologous immune serum, but fails to give a skin test in tuberculous animals.

Long and Seibert [6] have always maintained that the active principle of tuberculin is of protein nature. They were able to separate active and inactive proteins from tuberculin produced in protein-free mediums. By complete saturation with ammonium sulfate all of the protein and all of the skin reactive principle of tuberculin could be removed. They separated this precipitate into three fractions: a water soluble heat coagulable protein; a noncoagulable alkali soluble, water insoluble protein; and a noncoagulable water soluble protein. The first fraction possessed little activity, the second none, but the third was markedly active. In a later paper [7] they separated the precipitate thrown down by complete saturation with ammonium sulfate into a water insoluble, noncoagulable protein; a water soluble noncoaglable protein; and a water soluble coagulable protein. In this paper they state that the first product manifests little activity while the two water soluble fractions are about equally potent. The water soluble noncoagulable fraction dialyzes through animal membranes. They state "An active dialyzable prosthetic group is gradually loosened from the protein molecule during continued treatment with $(NH_4)_2SO_4$ or mild heat. On the other hand it may be interpreted to mean that some of the very first cleavage products of protein, namely the large proteose molecules which will just dialyze are still active and therefore responsible for the activity in diffusates." This last interpretation is the most probable and finds support in the data which show that these active diffusates usually give a pink biuret reaction. The same workers [8] have also shown that acetic acid precipitates an active protein. The isoelectric point of this active fraction is P_H 4.4, but according to Long and Seibert the neutralized supernatant even after complete precipitation with acetic acid gives a strong tuberculin reaction. They suggest that tuberculin is probably composed of more than one protein, each one separating out at a different isoelectric point. They state [9] that the skin reacting substances does not pass through dialyzing membrances, fish bladders or parchment, although a substance giving a positive Molisch test dialyzed to some extent, indicating that this carbohydrate is not especially associated with the skin reacting material. But, as previously stated, when the protein substance

[6] J. Am. M. A., 1925, 85, p. 650.
[7] Am. Rev. Tuberc., 1926, 13, p. 408.
[8] Ibid., p. 398.
[9] Ibid., p. 404.

obtained by complete saturation of tuberculin with $(NH_4)_2SO_4$ is fractionated, it was found [7] that considerable active substance does pass out of the membrane when the active water soluble noncoagulable fraction is dialyzed.

Eberson [10] prepared from O. T. acetyl and benzoyl derivatives of the methyl alcohol insoluble fraction and an ether soluble X fraction. All of these were capable of eliciting skin reactions in tuberculous guinea-pigs. These derivatives furthermore have definite sensitizing properties "since one or more injections of any one of the preparations suffice for the development of skin reactions in response to intracutaneous tests made with the homologous or heterologous derivative or with O. T." Likewise he [11] has been able to separate three active principles from tuberculins prepared with synthetic nonprotein mediums. One of these, the ether soluble fraction, gives none of the tests for protein. These fractions when injected subcutaneously sensitize normal guinea-pigs so that typical skin reactions are produced by the homologous or heterologous derivative when tested out one week after the last injection.

Since it has thus far been impossible to grow Br. abortus on a non-protein medium and since the preparations made from meat infusion or Liebig's broth cultures are very often either toxic or not very potent we proceeded to fractionate cell solutions prepared from whole bacteria by acetic acid precipitation. The procedure followed and outlined in detail in the following pages was one suggested by Zinsser but slightly modified in the series of experiments reported. Zinsser dries and then grinds his organisms before extraction. This procedure is omitted. Instead a very heavy alkalinized suspension of the living organisms is frozen and thawed and then shaken for from 2 to 4 hours until the bacteria are completely broken up. The fractions are then prepared from the bacteria-free filtrate.

Preparation of Powdered Bacterial Protein.—The powdered bacterial proteins used as control suspensions were prepared in the usual way; namely, the bacterial suspension removed from the surface of large blake bottle agar cultures was washed three times in 0.85% NaCl solution then twice in absolute alcohol and twice in water-free ether. The bacteria were then ground in a mortar and preserved in the dark in a desiccator. The day the tests were made the powdered material was weighed out, so that the solution would contain 5 mg. of dried substance to 10 cc. of salt solution, placed in a mortar and triturated with the requisite amount of salt solution. Four drops of N/10 NaOH were added to each 10 cc. of suspension to bring the cells into solution. These antigens were prepared fresh for each test.

[10] Am. Rev. Tuberc., 1925, 10, p. 681.
[11] Ibid., 1926, 13, p. 454.

Preparation of Cell Solutions Abortin A.—One cc. of Br. abortus culture, strain 80, originally isolated in this laboratory from certified milk, grown for three days in 5% glycerol Liebig's extract broth was spread on the surface of 5% glycerol tryptic blood digest agar contained in large blake bottles. The cotton plugs were dipped in paraffin, and the cultures incubated at 37 C. for three days. The growth was washed off carefully with 0.85% NaCl solution centrifugalized and washed three times in a large volume of salt solution. The suspension was placed in the ice chest over night, washed again the next morning, and resuspended in distilled water. The heavy emulsion was frozen and thawed 32 times (freezing mixture NaCl + crushed ice, approximately — 21 C.). Since the organisms did not seem to be entirely dissolved a part was made alkaline with N/1 NaOH. The emulsion became very viscous and slimy. N/1 HCl was added until the emulsion was no longer viscous. When acidified the supernatant was opalescent and a curdy precipitate separated. Enough alkali was again added to bring the reaction to P_H 9.2. After freezing and thawing another six times the smears of the suspension showed that the organisms were entirely dissolved. This suspension was then centrifugalized, then neutralized (P_H 7.0). with HCl. The following day the slightly opalescent fluid was passed through a Berkefeld filter and 10% acetic acid was added to the clear filtrate until a white precipitate was formed. The reaction was approximately P_H 4.0. After centrifugalizing the addition of another drop of acetic acid brought down more precipitate. This was again sedimented by centrifugalization; further addition of acetic acid to the clear supernatant did not produce any more turbidity. This supernatant was poured off. The precipitate was redissolved in distilled water and reprecipitated with 10% acetic acid; this procedure was repeated four times to insure the separation of all traces of "residue antigen" from the "nucleoprotein" fraction. The reaction of a portion of the acid supernatant was adjusted to P_H 7.0 and saved. The remainder was boiled for four minutes when the clear solution became opalescent. After filtering the hot solution through a Berkefeld filter the reaction was adjusted to P_H 7.0. On cooling another slight precipitate, probably Bence-Jones protein, separated from the clear solution. This was removed by decanting the clear supernatant.

Zinsser and Mueller [12] state that "this so-called nucleoprotein never seems to go into complete solution, always remaining opalescent, and if filtered through Berkefeld filters shows considerable loss, indicating that such so-called nucleoprotein solutions are really suspensions." The so-called nucleoprotein fraction of abortin, however, forms clear solutions in a slight excess of alkali, which upon neutralization remain clear and are as active as the original solution from which they were obtained.

The fractions in this and the following preparations are designated as follows: fraction 1, unfiltered cell solution; fraction 2, filtered cell solution; fraction 3, neutralized supernatant fluid after removal of the acid precipitable protein; fraction 4, neutralized solution of the acid precipitable protein; fraction 5, residue antigen, the neutralized filtrate obtained after boiling and filtering fraction 3.

[12] J. Exper. Med., 1925, 41. p. 159.

Preparation Abortin C.—These fractions were prepared from a portion of the original suspension used in the separation of the A fractions. The frozen and thawed organisms were suspended in 0.4% NaCl instead of distilled water. Enough N/1 NaOH was added to bring the reaction to P_H 9.2. The alkaline suspension was shaken for an hour, neutralized and centrifugalized. The procedure for the separation of the fractions was the same as described for preparation A with the exception that 0.85% NaCl solution was used for resuspending the precipitates. Furthermore the nucleoprotein fraction was reprecipated only two times, the first time at P_H 4.25, the second at P_H 4.4.

Preparation Abortin D.—Br. abortus strain 80 was grown for five days on 5% glycerol peptic digest agar. The growth from seven large blake bottles was washed off in 0.85% NaCl solution centrifugalized for 1½ hours, washed three times in fresh salt solution and then resuspended in about 80 cc. of salt solution. The reaction was adjusted to P_H 9.2 with N/1 NaOH and during a period of four hours frozen and thawed about seven times. The viscous suspension was neutralized, allowed to stand on ice over night and without changing the reaction frozen and thawed seven times on the following day. The

TABLE 1

DETERMINATION OF THE ISOELECTRIC POINT OF THE ACTIVE ACID PRECIPITABLE ABORTUS PROTEIN

	Tubes	Cc. 10% Acetic Acid Added	Turbidity	P_H
Trial 1	1	0.05	+	4.4
	2	0.15	+++	3.6
	3	0.30	+++	3.4
	4	0.35	+++	3.3
	5	0.65	+	3.2
	6	0.75	+	3.2
	7	1.35	±	3.0
Trial 2	1	0.05	+	4.4
	2	0.20	++	3.6
	3	0.30	+++	3.4
	4	0.65	+	3.2
	5	1.0	+	3.2
	6	1.8	+	3.2
	7	2.5	±	3.0

organisms in smears looked slightly amorphous. After centrifugalizing the suspension the opalescent supernatant was poured off, the sediment was resuspended in 30 cc. salt solution made alkaline (P_H 9.2) with N/1 NaOH and shaken for two hours. After neutralizing the suspension was centrifugalized and the opalescent supernatent was added to the first lot of bacterial solution. The liquid was then passed through a Berkefeld filter. Forty cc. of the clear slightly yellow tinged filtrate was fractionated with acetic acid, the remainder was saved and the protein precipitated by ammonium sulfate saturation.

Before fractionating the isoelectric point was determined by comparing the degree of turbidity of the solutions at different hydrogen ion concentrations. The P_H determinations were made colorimetrically—the values tabulated in table 1 are therefore only approximate.

Results shown in table 1 indicate that the isoelectric point of the acid precipitable protein lies between P_H 3.4 and 3.6. The acid precipitable fraction 4 D was therefore precipitated at P_H 3.5. The sedi-

mented fraction was redissolved in salt solution made slightly alkaline with N/10 NaOH. This procedure was repeated three times. The last precipitate was dissolved in salt solution and the reaction adjusted to P_H 7.0. A part of the acidified supernatant (fraction 3) was boiled for four minutes and passed, while hot, through a Berkefeld V filter, neutralized and allowed to cool. A slight precipitate probably Bence-Jones protein again separated as a compact mass. The clear supernatant was poured off—this was the "residue antigen" fraction 5 D.

The final volume of all of the protein fractions was, as far as possible, made up to the original volume from which they had been precipitated. The final volume of the residue antigen was of necessity in slight excess of the original volume of the whole cell suspension due to the addition of alkali during the final process of neutralization. This, however, never exceeded 5 to 10% of the original volume.

Precipitation of Protein with Ammonium Sulfate.—Fifty cc. of the autolysate used in the fractionation of preparation D was saturated with ammonium sulfate and allowed to stand over night at room temperature. The precipitate was separated from the liquor by centrifugalization and resuspended in 25 cc. 0.85% salt solution. A few drops of toluol was added and the solution dialyzed in a fish bladder for 48 hours. The protein solution, free from SO_4, was then passed through a mandler filter. An equal volume of 0.85% of NaCl solution was used to wash out the filter so that the final volume was approximately equal to the original.

On account of the addition of 0.25% tricresol to some of the solutions, the chemical tests could not be carried out on all the fractions prepared. Furthermore, tests showing the presence of protein mean little in the case of the abortins since the growths were not obtained from protein-free substrates. The tests carried out gave the following results: fractions 1 C gave a marked biuret and Adamkiewicz test; 3 C and 5 C gave faint biuret and Adamkiewicz tests, and 4 C gave a faint Adamkiewicz and marked biuret reaction; 2 D and 5 D gave a Millons and biuret but not Adamkiewicz reactions. However, the precipitate in Millons' test did not change color on heating. Fraction 4 D gave a biuret but not an Adamkiewicz test. Other protein tests could not be carried out on 4 D on account of the addition of tricresol. The ammonium sulfate precipitate likewise gave a positive biuret and Millons' reaction. It will be noted therefore that none of the fractions, not even the residue antigen, were protein-free.

Infection of the Guinea-Pigs.—Series 1: Large male guinea-pigs weighing from 600 to 800 Gm. were inoculated intramuscularly in the left hind flank with 0.2 cc. of a heavy emulsion of Br. abortus. In less than 10 days a large abscess formed at the site of inoculation which, in some instances, did not heal for a period of two months.

Series 2: Large male guinea-pigs weighing from 500 to 600 Gm. were inoculated intramuscularly with 1 cc. of an emulsion of the spleen of an infected guinea-pig, ground in about 30 cc. of salt solution. No local lesions developed.

Series 3: Large male guinea-pigs weighing from 500 to 600 Gm. were inoculated intraperitoneally with 1 cc. of the same emulsion used to infect series 2.

Series 4: Male guinea-pigs weighing from 300 to 400 Gm. were inoculated intramuscularly with 0.2 cc. of a heavy emulsion of Br. abortus.

Series 5: Small female guinea-pigs weighing about 250 Gm. were inoculated intramuscularly with 0.2 cc. of a moderately heavy suspension of Br. abortus.

Skin Tests.—The day before the skin tests were made the abdomen or sides of the animal were carefully shaven and the skin coated with pure olive oil. The following day the skin was cleansed with 70% alcohol and 0.1 cc. of the test substance or solution was inoculated intradermally. The reactions were measured after 18 to 24 hours, 40 to 48 hours, and again after 72 hours. Control tests with paratyphoid and abortus suspensions were made on the infected animals. All of the products were likewise tested on normal uninfected pigs. Control tests made with a cell solution and protein fraction of a guinea-pig paratyphous strain were also made on abortus infected animals. Since six or seven tests could be made on one animal it was an easy matter to compare the activity of the different fractions.

Reactions were judged according to the degree of local induration and inflammation, and the subsequent central necrosis of the lesion which was sometimes followed by scar formation. Transitory reactions usually persist for only 18 to 30 hours. mild and severe reactions become more intense after the first 24 hours, reach their height in about 40 hours, then retrogress and may persist for from 5 to 7 days. Reactions marked $+ + +$ or $+ + + +$ are of the latter type.

The skin reactions are designated in all tables as follows:

0 No reddening, no induration.

N. P. A slight mark from the needle prick (N. P.) or slight bruising.

± Reactions showing very slight reddening not measuring more than 0.8 cm. with no or only very slight induration.

+ Reactions showing slight reddening measuring 0.8-1.1 cm. and showing slight induration.

++ Reactions showing slight reddening measuring 1.0-1.5 cm. and showing only slight induration (a mild reaction).

+++ Reactions more markedly hyperaemic, measuring more than 1 cm. sometimes developing a central necrotic area and showing induration.

++++ Reactions showing marked reddening, measuring more than 1.0 cm. (usually 1.5-2.0 cm.) becoming markedly indurated and showing a central necrotic area usually 0.4 cm. in diameter (+++ and ++++ are definite severe reactions).

Results from the first series of animals inoculated and summarized in table 2 show that fractions 1, containing cell debris as well as the soluble cell substance, and 2 containing only the soluble cell solution, elicit

TABLE 2

Skin Reactions (at 30 to 44 Hours) on Guinea-Pigs Infected with Br. Abortus

Guinea-pig No.	Days In-fected	Paraty-phoidin* Suspension, 5 Mg. in 10 Cc.	Abor-tin* Suspension, 5 Mg. in 10 Cc.	Prep-aration Tested	Abortin Fractions					Remarks and Necropsy Findings
					Frac-tion 1	Frac-tion 2	Frac-tion 3	Frac-tion 4	Frac-tion 5	
1	15 and 25	0	0	A	0	0	0	0	0	
	49	0	±	C	±	0	0	+++	0	
	103	..	..	C	Hemorrhagic purpura dead in 15 hours					Joint involvement and marked abortus lesions at necropsy
2	25	0	±	A	+	..	0	±	0	.
	49	0	++	C	+++	+++	+	+++	0	
	103	..	..	C	+++	+++	+++†	++++	±	Fraction 5 negative in 72 hours. Others +++. Marked joint involvement
3	15	0	0	A	±	0	0	+++	0	
	25	0	±	A	++	..	0	+	0	
	49	..	..	C	Hemorrhagic purpura. Sick in 10 hours. Died 3 days after skin tests were made					Joint involvement and marked Br. abortus lesions
4	15	0	0	A	±	0	±	±	0	
	25	0	+	A	+	..	0	+	0	
	49	0	..	C	Hemorrhagic purpura				0	Slight joint involvement and marked Br. abortus lesions
5	15	0	0	A	0	±	0	0	0	
					25 days (−)					
	25	0	0	A	0	..	0	0	0	
	49	0	±	C	++	++	0	++	0	Joint involvement and marked Br. abortus lesions
6	15	0	0	A	±	0	0	++	0	
	25	0	0	A	+	0	0	+	0	
	49	0	0	C	+	+	0	+	0	
	103	..	..	C	++	+++	+++†	+++	0	Marked joint involvement
CONTROLS: UNINFECTED GUINEA-PIGS										
Tested on 15th day										
7 and 8	..	0	0	A	0	0	0	0	0	
					(guinea-pig 7‡)					
Tested on 25th day										
7	..	+	0	A	0‡	..	0	0	0	
8	..	+	±	A	+	..	0	+	0	
9, 10 and 11		0	0	A	0	..	0	0	0	
Tested on 49th day										
10, 11, 12 and 13	..	0	0	C	0	0	0	0	0	
14	..	0‡	0‡	C	0	0‡	0‡	0	0	
Tested on 103d day										
15	..	..	..	C	+	0	0	0	0	
16 and 17	..	..	..	C	0	0	0	0	0	

* Abortin preparation 1 was used on the 15th day, preparation 2 on the 25th and 49th days.

† This bottle had accidentally become contaminated with fraction 4 C.

‡ Indicates a very slight initial reddening and occasional slight induration which disappeared in a few hours. Other control guinea-pigs were uniformly negative at 18 to 24 and 30 to 48 hours.

marked skin reactions in infected animals. Judging from the reactions in normal animals the primary toxicity of these preparations is low. Fraction 3, the unheated residue freed from acid precipitable protein and fraction 5 the heated and filtered "residue antigen" are both incapable of detecting skin sensitization while the acid precipitable protein, fraction 4, is just as efficient as 1 and 2 if not more so in detecting skin sensitivity. The primary toxicity of these preparations is remarkably low. For this reason if for no other the value of this antigen is indicated. The fraction which Zinsser calls the "residue antigen" never gave a reaction, nor did fraction 3 excepting in the two instances noted when it was known to have been accidentally contaminated with fraction 4. Tests made on abortus infected animals with D fractions and shown in table 3 confirm these results.

TABLE 3

Skin Reactions with Abortus Preparations on Guinea-Pigs Infected with Br. Abortus

Guinea-pig	0.85 % NaCl, 24, 42, 68 Hours	Fraction 2 D (Filtered Dissolved Cells) Hours			Fraction 3 D (Unheated Residue Antigen) Hours			Fraction 4 D (Acid Precipitable Fraction) Hours			Fraction 5 D (Residue Antigen) Hours		
		24	42	68	24	42	68	24	42	68	24	42	68
18	0	0	++	++	0	±	±	0	+++	+++	0	0	0
19	0	+	++	+++	0	0	0	+++	++++	++++	±	±	±
20	0	..	..	..	0	0	0	+	+	++++	0	0	0
Control	0	±	0	0	0	0	0	+	±	±	0	0	0
Control	0	±	0	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0	0	0	0

The conflicting results reported in the literature which endow the residue antigen of tuberculin with the property of eliciting skin reactions in sensitized animals may on the one hand be due to the incomplete removal of the active protein or to the presence of more than one such protein. On the other hand the acid precipitation may carry down with the protein as an adsorption product nonprotein substances which can be dialyzed from the acid precipitate. This possibility in the case of abortin, although not probable, since the protein substance is prepared by repeated purification from alkalinized solutions, should be borne in mind.

Seibert and Long in a recent paper [7] endow the precipitate thrown down after completely saturating the solution with ammonium sulfate with the power of eliciting skin reactions in tuberculous guinea-pigs and points out that the dialysate in many instances contains skin reacting substances. The dialyzed solution of the precipitate thrown down after completely saturating the filtered abortin cell solution likewise produced

skin reactions in abortus infected animals. The dialysate, however, was not tested. Until such experiments are carried out with abortus protein prepared from organisms cultivated on nonprotein mediums the exact nature of the active substance cannot be stated definitely.

Control tests made with slightly alkalinized suspensions of ground and dried paratyphoid and abortus preparations showed that the autolysate and protein fraction gave a better reaction than the abortin suspension and that the reaction was specific. To further control the specificity of the reaction it was thought advisable to test the abortin infected animals with a paratyphoid solution made in the same manner as the abortin solution. The organism in this instance were grown on the synthetic mediums used by Long and Seibert [13] for the cultivation of tubercle bacilli. Glucose was substituted for glycerol, and a very luxuriant growth was obtained in 48 hours. The protein precipitate in this instance was considerably smaller than the abortin precipitate— the final volume was therefore made up to one-third the original volume. Fraction 2, the filtered autolysate, reacts with Millons' reagent and gives a definite biuret test; fraction 4 gives a doubtful biuret reaction, and a yellowish precipitate with Millons' reagent which does not change color on heating. Infected guinea-pigs were tested with these and the corresponding abortin fractions. The results are tabulated in table 4.

Slight nonspecific reactions were noted in this test probably due to the markedly sensitized state of these animals. The reaction to the heterologous protein regressed in 42 hours and in 70 hours it was entirely negative. The reaction to the homologous cell solution and protein however increased in intensity up to the 70th hour and remained so for several days. The atypical results shown by guinea-pig 20 will be discussed later.

Dilutions of fractions 2 and 4 tested out on infected guinea-pigs showed that in a susceptible animal a 1:10 dilution of the original solution will give a definite reaction although the degree of inflammation and induration is not so marked as in the original undiluted preparation. The same holds for fraction 4. The strength of the reactive substance will naturally vary with every new lot of cell solution prepared. Since no attempt has been made to obtain the active substance in a dry state, 0.1 cc. of the test solution, made in the course of preparation equal in strength to the original volume from which it was obtained, was inoculated intradermally. This amount chosen arbitrarily was, as shown in the preceding tables, sufficient to give a marked skin reaction and

[13] Am. Rev. Tuberc., 1926, 13, p. 393.

TABLE 4

SKIN REACTIONS WITH PARATYPHOID AND ABORTIN ANTIGENS ON GUINEA-PIGS INFECTED WITH BR. ABORTUS

Guinea-pig	Days Infected	To 0.85% NaCl Solution Control Read after 18 to 90 Hours	To Paratyphoidin Fraction 2 Hours					To Paratyphoidin Fraction 4 18 to 90 Hours	To Abortin Fraction 2 D Hours					To Abortin Fraction 4 D Hours				
			18	24	42	70	90		18	24	42	70	90	18	24	42	70	90
18	136	0	±	±	±	N.P.	0	0	+++	+	++	+++	+++	+	+	++	+++	+++
19	136	0	± sl. disc.	no disc.	no disc.	0	0	0	+++	+++	++++	++++	++++	+++	+++	++++	+++++	++++
20	136	0	trauma + 0	+	0	0	0	0	+++	+	++	±	±	+++	+	++	N. P.	N. P.
21	136	0	±	+	±	0	0	0	+++	+++	++++	++++	+++++	+++	+++	++++	+++++	++++
3 controls	..	0	0*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* One control gave slight ± reaction.

was entirely innocuous or produced only a slight transitory initial reaction in normal animals. The approximate strength of the reagent may in the future be roughly standardized by using standard suspensions to prepare the cell solutions. In the last two lots (C and D) prepared, this was approximately the case.

The atypical results shown by guinea-pig 20 (table 4) may be due to desensitization brought on by repeated skin tests at comparatively short intervals of time or to a gradual loss of skin sensitivity with progressing infection. Results shown in tables 2 and 5 indicate that animals may be retested at monthly periods with impunity but when shorter intervals of time are chosen, two to three weeks periods, the skin reactions are apt to be weak or entirely negative. That the weak reactions produced by the solution of protein precipitated by ammonium sulfate saturation and recorded in table 5 were not due to lack of activity

TABLE 5

Repeated Skin Tests on Guinea-Pigs Infected with Br. Abortus

Guinea-pig	Date Tested	Preparation Used	Skin Reaction
19	8/18/26	D	++++
	9/ 1/26	Solution of ammonium sulfate precipitate	++
	9/21/26	D	++++
20	7/14/26	4C	+++
	8/18/26	D	+++
	9/ 1/26	Solution of ammonium sulfate precipitate	++
	9/21/26	D	++

of the test solution was shown by the marked reaction observed in an infected animal tested for the first time with an abortin. The desensitization in the instances recorded probably persists for about three weeks as indicated in the recorded results.

Another of several phenomena of hypersensitiveness encountered was shown by guinea-pig 20 when tested for the fourth time. This animal, in which the skin reaction was very weak, gave a typical allergic conjunctivitis thus indicating that the animal was highly sensitized. The same reaction was noted in two other infected animals. In such instances a nonpurulent edema of the eyelid was noted 18 hours after the skin test was carried out. The edema was at its height in 18 to 24 hours and was markedly decreased in 40 hours and almost normal in 70 hours. However, even then a moderate dry exudate persisted on the eyelid. In all three instances only one eye was involved.

Shock following the intracutaneous inoculation of the abortin preparations was frequently encountered. Three of six infected animals (table 2) retested from three to four times and two from the same series tested at the same time but, for brevity sake, not included in the protocol, became manifestly sick, and developed over the entire abdomen an urticarial rash resembling hemorrhagic purpura. They succumbed after varying periods of time ranging from 10 hours to 3 days. The following case is typical: within 10 hours after the intracutaneous test one guinea-pig showed signs of extreme weakness, lay on its side, breathing became labored and the abdominal muscles were very much relaxed. When handled the animal made violent contractions with its forelegs; if left alone it lay quietly on its side. Within two hours the animal was found dead. The animals, when sick felt cold, those that remain ill for a longer period of time become listless and stay huddled together in the cage. The skin at the site of inoculation and usually extending over a large area turns to a brown color then takes on a reddish purple hue. At necropsy it is seen that all of the capillaries in the skin are markedly distended. In other instances the anaphylactic symptoms are noted but the skin changes are not so striking. Instead the skin reactions remain negative and the animal dies probably of anaphylactic shock. Similar manifestations have been noted in tuberculous guinea-pigs although the shock after the intracutaneous injection of the antigen is not so common. Small amounts of autolysate will likewise produce the same result. Another guinea-pig after receiving an intracutaneous injection with 0.11 cc. of fraction 2 C showed hemorrhagic purpura and died within 40 hours after the intracutaneous test was carried out. Three of four other guinea-pigs inoculated intracutaneously with 0.1 cc. of each of the C fractions showed definite symptoms in less than 15 hours were dead in from 15 to 24 hours, while the fourth died in 48 hours.

Tryptic Digestion of Skin Reactive Substance.—To demonstrate that the acid precipitable substance which possesses the skin reactive principle and is capable of producing systemic allergic reactions in infected pigs is of protein nature, the following experiment was carried out.

To 4.5 cc. of fraction D was added 0.5 cc. of Bacto-trypsin.* To another 4.5 cc. portion the same amount of boiled enzyme was added. After the addition of a few drops of toluol the mixture was incubated at 37 C. for 24 hours, then heated at 60 C. for 30 minutes and, together with the unheated and heated controls, tested in 0.1 cc. amounts on infected and normal guinea-pigs. The results are summarized in table 6.

* "Difco" standardized.

The data shown in table 6 indicate that the active principle was destroyed by tryptic digestion for 24 hours. One guinea-pig, infected for 218 days, and two of three others, infected for 148 days, reacted to the undigested portions but failed to react to the digested protein. The fourth reacted definitely only to the control incubated portion. One of the three guinea-pigs infected for 76 days did not react at all, the second of this series showed only mild reactions to the control untreated and unincubated fraction, while the third gave mild reactions with the untreated as well as the heated control but not to the digested fraction or incubated control. The skin reactions of these last three animals, as well as those shown by two guinea-pigs from the same series tested

TABLE 6

Effect of Tryptic Digestion on Skin Reactive Principle in Acetic Acid Precipitable Abortus Protein

Guinea-pig	Number of Days Infected	Fraction 4 D Unincubated Control Hours			Fraction 4 D Heated 60 C 30 Minutes Hours			Fraction 4 D + Difco Trypsin Incubated 37 C. 24 Hours Then Heated 60 C. 30 Minutes Hours			Fraction 4 D + Boiled Difco Trypsin Incubated 37 C. 24 Hours Then Heated 60 C. 30 Minutes Hours		
		24	48	67	24	48	72	24	48	72	24	48	72
6	218	++	+++	+++	++	+++	+++	N. P.	±	±	++	++++	+++*
21	148	++	+++	++	++	+++	+++	N. P.	±	±	++	++	+++*
22	148	++	+	+	N. P.	±	±	++	±	±	+++	+++	+ *
23	148	++	+++	+++	+	+++	+	±	N. P.	N. P.	+	+++	+++*
24	76	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	N. P. *
25	76	++	+++	0	0	++	0	+	±	±	0	N. P.	± *
26	76	++	+++	N. P.	++	+++	N. P.	0	0	N. P.	+	±	0 *
27	..	±	0	0	±	0	0	±	±	0	±	0	0
28	..	0	0	0	0	0	0	0	0	0	0	0	0

* Necropsy findings: marked abortus lesions.
Explanation of symbols on p. 459.

three weeks previously indicate that these animals were not highly sensitized, although severely infected as shown by necropsy findings.

Unfortunately a control consisting of unheated enzyme inactivated after 24 hours incubation at 37 C. was omitted. It is possible that the digested broth may have been responsible for the slight initial response of guinea-pigs 22 and 25. These two animals as well as 26 gave irregular reactions. Whether variability in sensitivity of different areas of the skin or other unknown factors are responsible for these irregularities has not been investigated.

DISCUSSION

The experiments recorded in the preceding pages show that skin reactions are elicited in abortus infected guinea-pigs by an acid precipitable fraction of the filtered cell solution of the homologous strain.

The problem to determine whether the activity of the fraction responsible for the allergic reactions is due to a protein substance or to an adsorption product carried down by the foreign protein suggested itself. Unfortunately it has been impossible to cultivate Br. abortus on nonprotein culture mediums, asparagin and ammonium lactate alone or in combination cannot be utilized as the sole source of nitrogen by this organism. Likewise the synthetic mediums used by Long and Seibert are unsuitable. It is obviously impossible to exclude traces of foreign protein contained in the culture medium from the cell solution. If the activity of the acid precipitable fraction were due to an adsorption product carried down during precipitation one would expect the original filtered fraction to be more potent than the purified product. However, this is not the case. Both the original and the acid precipitable fraction, the latter made up to the volume from which it was originally obtained, have the same activity.

Moreover, one would anticipate that at least the unheated neutralized supernatant, from which the acid precipitable protein had been removed, would retain a certain amount of activity. However, such a condition was not encountered. According to Long this is the case with tuberculin. He attributes the allergic properties of the supernatant, after removal of the acid precipitable protein to the activity of other proteins not removed by acid precipitation. Only complete removal of the protein by ammonium sulfate saturation takes out all of the active substance. It must be remembered that the allergens in the two instances are not the same. The tuberculins are prepared from filtrates of old cultures, the abortin from the cell itself. In all of the abortin cell solutions the metabolic products are removed as completely as possible by repeated washing in large amounts of salt solution. The complete removal of the active principle by acid precipitation may be attributed to one of several factors: the source of the active portion may influence its composition or its structure to such an extent that it is more easily precipitated; there may be in tuberculin a multiplicity of active substances, in the case of abortin only one such product exists, and again, in abortin it is possible that the strength of the original cell solution is such that the removal of a part of the active principle leaves the supernatant too weak to elicit skin reactions. However, the experiments which demonstrate the tryptic digestion of the acid precipitable substance furnish further evidence that the allergen is in all probability of protein nature.

CONCLUSIONS

Cutaneous hypersensitiveness in abortus infected guinea-pigs may be demonstrated by testing the infected animal intracutaneously with filtered cell solution of Br. abortus. The substance precipitated by acetic acid in the cold at P_H 3.5 to 4.0 elicits allergic reactions as effectively as the original solution. The neutralized supernatant, both unheated and heated, devoid of this acid precipitable substance, is ineffective. Digestion with trypsin destroys the activity of the acid precipitable protein.

AN INVESTIGATION ON THE PRODUCTION OF B. ABORTUS AGGRESSIN

S. J. Schilling and Wm. L. Bleecker

From the Experiment Station, College of Agriculture, University of Arkansas, Fayetteville

Investigations concerning means of active immunization of cattle against infectious abortion have centered chiefly about the use of bacterins and of vaccines derived from cultures of B. abortus; these products have proved to possess some antigenic value. Bacterial extracts and metabolic products, per se, of B. abortus do not appear to have been utilized in experimental work thus far reported. Among the latter group of substances we have formerly recognized the aggressins. This paper presents the results of experiments designed to determine whether B. abortus, under the conditions of the experiment, may be caused (a) to produce agressins. It is well to recognize that when we use the term aggressins we are speaking of substances, the existence of which many bacteriologists and immunologists do not recognize. Authors of recent text books on bacteriology and immunology with few exceptions [1] leave the existence of agressins as an open question,[2] deny their existence,[3] or ignore [4] them altogether. There are on the market today, however, with official recognition, "aggressins" for hemorrhagic septicemia, for blackleg, and for anthrax. These products are being used rather widely and undoubtedly have some immunological value. In view of these facts and because, as will be discussed in the succeeding paragraphs, there is some experimental justification for tentatively accepting the term "aggressin" this term will be used in this report.

In the development of the aggressin theory we find that the first recorded suggestion regarding their existence was made in 1884 by Salmon and Smith.[5] They said: . . . "the germs of such maladies are only able to multiply in the body of the individual attacked because of a poisonous principle or substance which is produced during a multiplication of these germs." Zinsser [6] cites that Bouchard in 1893 found

[1] Kolmer, J. A.: Infection, Immunity, and Specific Therapy, 1920. p. 104.
[2] Zinsser: Infection and Resistance. 1923, p. 17. Buchanan: Veterinary Bacteriology, 1922, p. 204. Park & Williams: Pathogenic Microorganisms, 1920, p. 242.
[3] Morrey: Fundamentals of Bacteriology, 1921, p. 288.
[4] Jordan: General Bacteriology, 1924.
[5] Proc. Biol. Soc., 1884, 6, p. 29.
[6] Infection and Resistance, 1923, p. 17.

substances in the cultures of virulent bacteria which seemed to reenforce their invasive powers, and that, in the same year, Kruse suggested that bacteria may secrete substances which paralyze the destructive properties of animal serum. Rosenau [7] in 1907 reported the extraction of substances from pneumococci which in their properties conform closely to those of aggressins; he however named these substances "virulins." The substance of the aggressin theory was formulated by Bail,[8] 1904, and is the expression of an interpretation placed on results which he obtained in experimental work. It may be briefly recapitulated as follows:

Aggressins are substances secreted by some pathogenic bacteria; contact with body tissues or fluids may in some instances be necessary for the secretion of aggressin.

Aggressins interfere with the natural defensive mechanism of the animal as by suppression of phagocytosis and thus bring about lowered resistance to infection; thus what might otherwise be a nonlethal infection is enabled to overwhelm the animal.

Aggressins act as specific antigens; they stimulate the animal body to produce antiaggressins which have the property of neutralizing aggressins.

The aggressin theory soon became the center of a controversy; experimental work designed to test its validity, and in considerable amount, was performed and published.

It is not the object of this paper to present the arguments which have been adduced nor to cite the experimental procedures which have been construed to support or to controvert this theory. It is desired, however, to indicate that Wassermann and Citron [9] who did most of the experimental work in refutation of the agressin theory agreed with Bail and the other proponents of that theory with regard to experimental results. They did not agree however in the interpretation of those results. What Bail conceived to be secretions of bacteria and named "aggressins" Wassermann and Citron held to be bacterial extracts analogous to toxins. The antigenic qualities of the substances were not disputed. It seems beside the point to place excessive emphasis upon the question whether secretions or extracts of bacteria are involved since our means of differentiating between them are so limited. This leaves the argument hinging upon the point that Bail held aggressins to be distinct substances worthy of separate classification, while Wassermann and Citron considered them to belong to the general group of exotoxins. Since the researches on aggressins as notably exemplified in

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[7] J. Infect. Dis., 1907, 4, p. 285.

[8] Centralbl. f. Bakteriol., I. O., 1904, 37, p. 270.

[9] Citron: Centralbl. f. Bacteriol., I. O., 1906, 41, p. 230.

the case of anthrax aggressins showed that these substances possessed no demonstrable degree of toxicity, there may still be some room at this stage of our knowledge to tentatively assign them with a distinctive designation. It would be hazardous to venture to say what our concept with regard to the nature of agressins ultimately will be. It is enough for the purposes of this paper to recall that unrefuted experimental evidence exists to indicate that under proper conditions some species of bacteria may be made to yield relatively nontoxic products of their own metabolism which when used on animals may induce a state of fortified resistance to infection, and that these substances when injected into animals along with some of the organisms from which they were derived is followed by an intensification of the resultant infection. For reasons given above then these products will here be called "aggressins."

TABLE 1

Amount of Exudate and Relative Rate of Disappearance of Bacilli

Guinea-Pig	Amount Injected, Cc.	Dead in Hours	Exudate Recovered, Cc.	Bacilli in Peritoneal Exudate
1.............	5.0	20	2.2	±*
2.............	5.0	26 (killed)	3.2	±*
3.............	5.0	24	10.0	++
4.............	10.0	18	12.0	++
5.............	10.0	16	5.0	++
6.............	10.0	15	10.0	+++

* Sign ± indicates that only disintegrating forms were found.

EXPERIMENTAL DATA

A 48 hour growth on pork agar of virulent B. abortus was suspended in physiologic salt solution. Its density compared with that of tube 3 of a McFarland nephelometer. This suspension was inoculated in quantities of 5 cc. into the peritoneal cavities of each of three guinea-pigs. Three other guinea-pigs received 10 cc. of this suspension intraperitoneally. The animals were closely observed and necropsied immediately at their death. At necropsy the fluid found in the peritoneal cavity was aseptically removed and measured with sterile pipets. The exudate was placed in sterile cups and centrifuged at high speed. The clear supernatant fluid was removed by means of a pipet and enough phenol was then added to the exudate to made a 0.5% solution. After addition of the phenol the material was sealed in sterile glass ampoules. Smears were made from the untreated peritoneal exudate, stained and examined under the microscope for the purpose of studying the organisms therein contained. Table 1 shows the amount of inoculum received by each guinea-pig, the length of time it lived after inoculation, the amount of exudate recovered, and indicates by plus marks the relative number of B. abortus organisms present in the peritoneal cavity as judged microscopically by a comparison of stained preparations from peritoneal exudate.

The exudate from guinea-pig 4, treated as described above, was used in further work. Since it represented a fluid culture of B. abortus in the presence of living tissue and body fluids, it was anticipated that if

aggressins are produced by the organism in question they should be present in the exudate and their presence should be capable of demonstration. Tests for the presence of aggressins in the exudate were made by following experimental work in which aggressin action, i. e., acceleration or intensification of infection, might be observed. Other tests for their presence were undertaken under conditions in which if the exudate contained aggressins, formation of antiaggressins with consequent fortification of resistance of the test animals might occur.

Aggressins.—Tests for the presence of aggressins in the exudate of guinea-pig 4 under circumstances which could reveal their presence by aggressin action were made as follows:

Guinea-pigs 7 and 8 were each given 0.5 cc. of the exudate; guinea-pigs 9 and 10, 1.0 cc. each, and guinea-pigs 11 and 12, 2 cc. each, intraperitoneally. These animals, as well as the controls, 13, 14, 15, and 16, were inoculated subcutaneously at the same time with 1.0 cc. of a suspension of a young culture of B. abortus in physiologic salt solution. Thus the presence of aggressins in the exudate should make itself manifest by an earlier or a more severe development of the inoculation disease in those animals receiving exudate; the disease should also show more virulence in the guinea-pigs receiving the larger quantities of exudate. Two weeks after inoculation, necropsies were performed on exudate treated guinea-pigs 7, 8, 11, 12, and control guinea-pigs 13 and 14. At this time the guinea-pigs were weighed, chloroformed, the spleens carefully removed, measured, weighed, and cultured. Uninoculated guinea-pigs 17, 18, 19, and 20 were also necropsied in order to secure data on normal guinea-pigs. The results of this work, are given in the first part of table 2. Guinea-pigs 9, 10, 15, and 16 were necropsied four weeks after inoculation, when data was gathered on them, as in the group which was necropsied earlier. These findings are presented in the second part of table 2.

It is recognized that B. abortus tends to localize and produce lesions in the spleen of the guinea-pig. Therefore this organ was in each case subjected to special examination. The first part of table 2 shows that the mass of spleen and the proportion of weight of spleen to body weight did not differ significantly as between guinea-pigs receiving live cultures alone and those receiving exudate and live cultures simultaneously. Furthermore, guinea-pigs 11 and 12 receiving 2.0 cc. of exudate showed no more extensive progress of the inoculation disease than did guinea-pigs 7 and 8, receiving 0.5 cc. exudate; this also argues against the possibility of aggressin action being involved. The fact that B. abortus was recovered from all animals but one indicates that the live culture used was of sufficient virulence and was injected in sufficient quantities to yield a test. The sizes of spleens as determined by linear measurements and the weight of spleens two weeks after inoculation with B. abortus were found not to vary to any extent from the corresponding

data secured from normal guinea-pigs. The results of experimental work as presented in table 2, then, may be taken to indicate that the exudate which may be recovered from the peritoneal cavity of the guinea-pig after infection with large quantities of B. abortus suspension does not contain aggressin.

The remaining guinea-pigs were necropsied four weeks after treatment with exudate and live cultures (table 2, 2nd part). Therefore, a later state of the inoculation disease was found in them. However, there was no clear cut indication that the administration of exudate to guinea-

TABLE 2

Effect of Exudate from Guinea-Pig 4 (Table 1)

Data on Guinea-Pigs Necropsied Two Weeks After Inoculation, and on Controls

Guinea-Pig	Inoculated				Data on Spleens			
	Received Exudate, Cc.	Live Culture, Cc.	Necropsy, at Weeks	Gross Weight, Gm.	Weight, Gm.	% Body Weight	Size of Spleen, Mm.	Culture of Spleen
7	0.5	1.0	2	307	0.50	0.162	23.0 × 13.5	+
8	0.5	1.0	2	328	0.53	0.186	22.5 × 10.0	+
11	2.0	1.0	2	397	0.62	0.156	26.5 × 14.0	0
12	2.0	1.0	2	372	0.66	0.177	26.0 × 14.0	+
13	...	1.0	2	298	0.88	0.295	26.0 × 15.5	+
14	...	1.0	2	386	0.65	0.168	26.0 × 14.0	+
17	...	...	..	365	0.88	0.241	26.0 × 16.5	0
18	...	...	..	379	0.57	0.150	27.0 × 14.0	0
19	...	...	..	358	0.41	0.113	22.0 × 17.5	0
20	...	...	..	308	0.47	0.152	22.0 × 17.5	0
Data on Guinea-Pigs Necropsied Four Weeks After Inoculation								
9	1.0	1.0	4	407	1.25	0.210	31 × 17	+
10	1.0	1.0	4	414	1.29	0.311	30 × 17	+
15	...	1.0	4	495	1.15	0.232	33 × 16	+
16	...	1.0	4	385	1.70	0.441	36 × 23.5	+
Average of Controls 17 to 20								
				352.5	0.58+	0.164	24.25 × 16.37	0

0 = negative culture, and ..., no test made, in all tables.

pigs 9 and 10 had accelerated or intensified the pathologic process caused by B. abortus infection, as compared with guinea-pigs 15 and 16 which did not receive exudate. These results then, may be taken to corroborate those presented in the first part of table 2, and give further evidence that aggressins are not produced by B. abortus, at least under the conditions of the experiment.

Antiaggressins.—The test for the presence of aggressins in the exudate of guinea-pig 4 by determining whether it possessed an immunizing action was conducted as follows:

Two guinea-pigs, 21 and 22, were injected intraperitoneally with 2.0 cc. each of the exudate in question. Two weeks later they and two untreated guinea-pigs, 23 and 24, were inoculated subcutaneously with 1.0 cc. of a suspension in normal salt solution of a young culture of virulent B. abortus. Two weeks after inoculation, these guinea-pigs were necropsied and observations made to determine whether

the exudate-treated animals were or were not protected against the subsequent inoculation. Any evidence of such protection could be considered as presumptive evidence of the presence of aggressins in the exudate which had induced the production of antiaggressins in the treated guinea-pigs, which in turn would be expected to confer some degree of immunity. The body and spleen weights, spleen cultures and measurements were secured as before. The data secured in this manner is presented in table 3.

Daily weights were taken of guinea-pigs 21, 22, 23, and 24, commencing at the time of administration of virulent cultures and continuing up to the date of necropsy. There was no significant difference in the behavior of the weights of the animals to indicate that the injection of exudate into guinea-pigs 21 and 22 had modified the result in any manner. The weights therefore are not presented.

B. abortus was isolated from the spleens of all guinea-pigs inoculated. This indicates that if any protection was induced in response to the administration of the exudate, that it was insufficient to afford complete

TABLE 3

Test for Aggressins in Peritoneal Exudate by Its Power to Stimulate Formation of Antiaggressins

Guinea-Pig	Received, Exudate, 5/8/26, Cc.	Inoculated Live Culture, 5/22/26, Cc.	Gross Weight, Gm.	Data on Spleens			
				Weight, Gm.	% Body Weight	Size of Spleen, Mm.	Culture of Spleen
			Necropsies performed, 6/6/26				
21	2.0	1.0	455	1.18	0.259	30 × 15	+
22	2.0	1.0	375	0.53	0.141	28.5 × 11	+
23	...	1.0	321	0.50	0.155	22 × 12	+
24	...	1.0	680	2.03	0.298	40 × 18	+
Average for 4 normal guinea-pigs...			352.5	0.58+	0.164	24.25 × 16.37	0

protection. In order to determine whether partial protection might be afforded, a further comparison may be undertaken. Guinea-pigs 21 and 24 had the most markedly enlarged spleens, and the highest rates of weight of spleen to the body weight of the animal. Since one of these was exudate treated and the other was not, this is an indication that the injection of exudate probably was without immunizing effect. In guinea-pigs 22 and 23, pathologic changes of the spleen had not advanced to an extent which could be determined by weight or by measurement. Since one of these was treated with exudate and the other was not, they present no evidence of protection following the administration of exudate.

SUMMARY

The injection of a suspension of virulent B. abortus into the peritoneal cavity of guinea-pigs resulted in the early death of the animals with accumulation of purulent exudate in that cavity.

Guinea-pigs after simultaneous injection with the centrifugated and phenolized exudate, and with a suspension of live cultures, presented at two and four weeks no more marked progression of the inoculation disease than guinea-pigs receiving live cultures only; animals receiving larger doses of exudate showed no more advanced pathologic changes than those receiving less. Thus no aggressin action was observed.

Guinea-pigs which were given injections with exudate and two weeks later were inoculated with live cultures showed no evidence of immunization. This also indicates that the exudate probably contained no aggressin.

MAGNESIUM AMMONIUM PHOSPHATE CRYSTALS IN AEROBIC CULTURES OF BRUCELLA ABORTUS AND BRUCELLA MELITENSIS

I. Forest Huddleson and O. B. Winter

From the Bacteriological and Chemical Sections, Michigan Agricultural Experiment Station, East Lansing

For several years we have observed the occurrence of a peculiar crystal formation in aerobic cultures of Brucella abortus and Brucella melitensis on a semisolid medium. Scudder [1] also has observed the same type of crystals in a medium of beef heart hormone base, agar and peptone. No similar crystal formation, however, has been observed by us to take place in uninoculated medium placed under the same conditions.

The study of crystal formation resulting from the growth of Br. abortus and Br. melitensis has been made on a culture medium composed of beef liver infusion, bacto-peptone, sodium chloride (c. p.), shredded agar, tap water and brom thymol blue indicator. Slants were prepared and sterilized, and the reaction of the medium adjusted to approximately P_H 6.6.

When slants of the medium are well seeded with Br. abortus or Br. melitensis left unsealed and placed in a 37 C. incubator, one will observe that as the growth and age of the culture increase, the P_H of the medium also increases. After the growth becomes visible on the surface of the slant, the color gradually changes from a greenish yellow to a faint green, and later at about the 72d hour of incubation, assumes a dark green color. While the above changes in the color of the growth are taking place, similar changes are taking place in the medium; first, just under the growth and later gradually extending downward. If the growth is of sufficient abundance to cover the slant, the maximum change in reaction will occur between the 72d and 120th hour of incubation. The final P_H of the growth and of the medium to a certain depth is near 7.4.

Accompanying the change in the reaction of the medium is the appearance of clear macroscopic crystals which increase in size with the increase of the P_H and the age of the culture. These crystals become visible to the eye about four or five days after the seeding of a fresh slant of the medium, first in the growth proper and later under the growth medium to a depth of from 1 to 5 mm. The size of the crystals varies from 1x1 mm. to 1x3 mm. The crystals begin to form when the medium attains a P_H of from 7.2 to 7.4.

If similar tubes of mediums are seeded with either of the organisms in question and the tubes are sealed or placed in an atmosphere in which 10% of the air is replaced by carbon dioxide the appearance of visible growth following incubation at 37 C. causes little or no change in the P_H of the medium. On the other hand, if the original P_H of the medium lies within a range of 6.8 to 7.4 the above conditions will bring about a gradual increase in the acidity of the medium. Under these conditions, there is no crystal formation. If the

[1] J. Bact., 1926, 11, p. 90.

tubes are now unsealed or removed from the carbon dioxid atmosphere, the medium and mass of growth will gradually become alkaline in reaction and crystals will make their appearance.

In order to determine the nature of the crystals and to throw more light on the chemistry of their formation, a sufficient number of them was removed on several occasions from the medium in which they grew and subjected to chemical and microscopical examination.

Since it was found practically impossible to free the crystals entirely from the surrounding medium, no attempt was made at a quantitative chemical analysis. However, qualitative chemical tests were made and some of the optical properties determined. Ammonia, magnesium, phosphoric acid and a trace of chlorine were found. (Undoubtedly the chlorine was present in the adhering medium.) The crystals were found to be insoluble in water, organic solvents, ammonia and dilute alkalies. They were soluble in dilute mineral acids leaving a sort of honeycomb residue which appeared like some of the adhered medium. After dissolving the crystals in dilute acid and then making the solution alkaline with either ammonia or a fixed alkali, they were reprecipitated. The optical properties of the crystals were found to be as follows: biaxial, positive, parallel extinction in the long direction, medium optical angle, refractive index = 1.503, and most of the crystals when not broken lay on a flat surface nearly normal to the acute bisectrix. When the crystals were dissolved in dilute acid on a micro slide and the solution made slightly alkaline, the precipitate which formed was amorphous or very finely crystalline at first, but soon x-like crystals separated out and on standing a little longer rectangular prisms resembling roofs of houses appeared.

The crystals are evidently magnesium ammonium phosphate, (Mg NH_4 PO_4 $6H_2O$), since qualitative chemicals tests on this salt are similar to those mentioned above, and its optical properties given in the literature [2]—biaxial, positive orthorhombic system, refractive index 1.502, optical angle 47° 30′, slow ray parallel to b-axis— are nearly the same. Also, when magnesium ammonium phosphate crystallizes on a micro slide under conditions of the technic for determining the microchemical reactions of the common elements,[3] the results are similar to those mentioned above.

DISCUSSION

It is common knowledge that the energy for the growth and multiplication of microorganisms is derived from the decomposition of proteins, amino acids, carbohydrates or salts. In the case of Br. abortus and Br. melitensis we have positive evidence that certain amino acids, present in the peptone and liver infusion are the chief sources of growth energy. One of the products of decomposition is ammonia. This product is responsible for the increase in the P_H of both the growth on the surface of the slant and the culture medium.

[2] Chamot: Elementary Chemical Microscopy. 1915, p. 287.
[3] U. S. Agric. Bull. 1108, 1922, p. 13.

Now, it is a well known fact that magnesium phosphate is present in nearly all animal tissue, especially the liver. The presence of ammonia in a medium containing magnesium phosphate, as in the above case, results in the precipitation of the salt as ammonium magnesium phosphate. The crystals become visible presumably from the fact that a semisolid medium retards their rate of growth to such an extent that they become large and well developed.

Crystals of the same nature and composition may be produced artificially, that is, in the absence of the growth of the organism by exposing the surface of petri dishes containing the solidified medium to ammonium hydroxide in an incubator at 37 C. Crystals in large numbers will make their appearance on the surface of the medium in less than 24 hours.

It is stated that when an inoculated tube of the medium is sealed or placed in an atmosphere of 10%, CO_2 the P_H does not increase, but may be observed to decrease slightly if the culture is incubated for several days. The reaction of the medium is controlled in the first case by the respired CO_2 of the organisms, and in the second case by the excess of CO_2 in the atmosphere in which the cultures were placed.

The formation of the salt in question appears to have more than a passing significance. We have observed repeatedly that the viability of the organism is greatly interferred with when ammonium magnesium phosphate is found in abundance. In other words its viability decreases as the salt increases.

It is also observed that old strains of both Br. abortus and Br. melitensis differ markedly in their ability to bring about the formation of this salt. It is necessary to transfer several strains every ten days in order to maintain their viability under aerobic conditions.

CONCLUSIONS

From our observations of the growth of Br. abortus and Br. melitensis on the medium described in this paper and from the examination of crystals, we made the following conclusions. The crystals are magnesium ammonium phosphate ($Mg\ NH_4\ PO_4\ 6H_2O$), and their large size and well developed shape are due to their slow formation in a semisolid medium (a gel).

The increase in the P_H of the medium and the separation of the crystals are due to the formation of ammonia produced by the activity of the organisms in question on the culture medium.

The formation of this salt interferes with the viability of Br. abortus and Br. melitensis.

THE SPECIFICITY OF THE WEIL-FELIX REACTION IN BRILL'S DISEASE

Leon C. Havens

From Laboratories of Alabama State Board of Health, Montgomery

The studies by Weil and Felix [1] of strains of B. proteus, isolated from cases of typhus, have produced a useful agglutination test for the diagnosis of typhus fever. Although the reaction is not biologically specific, many observers [2] attest its practical specificity in this disease. In the United States there has been little occasion for extensive use of this test, but in spite of this fact, criticisms of its specificity have appeared in the literature. Weeter and Motyca [3] have examined 814 serums for the presence of agglutinins for the X19 strain of B. proteus. It is stated that typhus could be excluded in each instance. They obtained positive results in a dilution of 1 : 100 with 26 serums, and in a dilution of 1 : 200 with three. Gilbert and Coleman [4] have described a case which gave a positive Weil-Felix reaction in a dilution of 1 : 1,600. They concluded that this reaction was not specific because the agglutinins were not destroyed by heating at 56 C. They were lost, however, at 62 to 65 C. for one hour. A final diagnosis was not made in this case, although the clinical description fits that of Brill's disease or mild typhus. The reasons for excluding this diagnosis are not entirely obvious.

There are two questions raised by these observations. One concerns the specificity of the test in the dilutions used (1 : 100). The second deals with the question of the thermolability of the agglutinins.

The Laboratories of the Alabama State Board of Health have had in the past three years a relatively extensive experience with the Weil-Felix reaction and it is felt that a discussion of this experience may help in evaluating the usefulness of this test, particularly in Brill's disease or mild typhus, as it exists in the United States.

Macroscopic Weil-Felix Test.—Technic: Serial dilutions of the serum were made from 1 :10 to 1 :2560 or higher. To 0.5 cc. amounts of the serum dilutions were added 0.5 cc. amounts of a suspension in normal salt solution of a fresh

[1] Feldärztliche Blätter der k. u. k. Armée, No. 11, 1926.
[2] Fairley, N. H.: J. Hyg., 1919, 18, p. 203; Montefusco, A.: Riforma Medica, 1919, 35, p. 782; Chambers, J. H.: U. S. Naval Med. Bull., 1922, 17, p. 211; Fletcher, Wm., and Lesslar, J. E.: Bull. Inst. M. Res. Fed. Malay States, 1926, No. 1.
[3] Southern M. J., 1925, 18, p. 650.
[4] J. Infect. Dis., 1925, 37, p. 559.

agar slant culture of B. proteus X19. The tubes were incubated for two hours at 37 C. and allowed to stand at room temperature overnight. The final titer of the serum was taken as that dilution showing definite agglutination the following morning.

Results: Since the early part of 1923, 1743 tests have been made; 139 of these gave positive results in dilutions of 1:80 or greater. All but five fell between 1:160 and 1:2500. One serum agglutinated in a dilution of 1:5,000 on the fourteenth day and one in 1:10,000 on the eleventh day. Agglutination in a dilution of 1:80 or higher has been considered specific, particularly in the presence of typical clinical symptoms, although, in most cases, a higher titer is obtained during the second or third week. In our experience, agglutination has never been obtained in dilutions greater than 1:50 except in cases with a characteristic Brill's disease syndrome.

The agglutinins for B. proteus X19 develop as a result of the disease process. A positive test has not been obtained earlier than the sixth day of the disease and in those instances where more than one specimen was obtained the titer invariably increased during the course of the disease. Table 1 gives the titers of the serums in the cases in which two specimens were obtained.

TABLE 1

Patient	Serum Specimen 1		Serum Specimen 2	
	Day of Disease	Titer	Day of Disease	Titer
1	6	80	12	640
2	10	320	14	1280
3	12	320	19	640
4	5	20	14	2560
5	5	0	14	5000
6	4	0	10	160
7	3	0	15	1280
8	7	0	14	80
9	12	0	18	1280
10	13	0	17	640

The ten cases in table 1 show a marked increase in the agglutinins from titers varying from 0 to 320 during the first week of the disease to titers of 80 to 5,000 during the latter part of the second, and during the third week. Cases 9 and 10 are noteworthy in that no agglutinins were present as late as the twelfth and thirteenth days of the disease, whereas on the eighteenth and seventeenth days, respectively, the tests were positive in high dilutions of the serums.

Three cases, which were found to be typical clinical cases of Brill's disease, failed to give a positive Weil-Felix test. One case gave a negative test on the seventh day, one on the tenth and the third on the seventeenth.

The Microscopic Weil-Felix Test.—Holt-Harris and Grubbs [5] have advocated the use of the microscopic technic with dried blood for rapid demonstration of suspicious cases of typhus fever. In the course of the investigation of the prevalence of Brill's disease in Alabama a microscopic Weil-Felix test was made on all specimens of dried blood submitted for the Widal test. Microscopic tests were made in 747 cases, using the same dilutions (aproximately 1:40 and 1:80) as for the Widal test. Forty-five of these showed definite agglutination in the higher dilution, but only four could be confirmed by subse-

[5] Pub. Health Rep., 1922, 37, p. 1675.

quent macroscopic tests and clinical histories. This experience throws doubt upon the value of the cicroscopic technic in the dilutions used. It may or may not be significant that twenty-one of the forty-five positive microscopic tests also gave a positive Widal reaction.

Effect of Inactivation of Serum: It has been shown that in virulent typhus the agglutinins for B. proteus X19 are relatively thermolabile, in contrast to the specific agglutinins produced in the rabbit by injections of B. proteus cultures. The exact degree of temperature required does not seem clear. Thus, Hamburger and Bauch[6] reported two cases, in one of which the agglutinins were destroyed by heating at 56 to 60 C., in the other at 60 to 65 C. for one-half hour. Csepai[7] reports destruction of these agglutinins at 63 C. and Werner and Leonann[8] at 65 C. Others have made similar observations. Kathe[9] found that the agglutinins in serums from thirty cases of typhus were not destroyed at 56 C., were diminished at 60 C., and in all but four were completely destroyed by heating at 65 C. for one hour.

Gilbert and Coleman[4] found that the serum from the case which they describe still agglutinated after heating at 56 C. but lost its agglutinins at 62 to 65 C. Hone and Bull[10] have described cases resembling Brill's disease in

TABLE 2

EFFECT OF HEAT ON X19 AGGLUTININS

Patient	Titers of Serums		
	Fresh	After 1 Hour at 56 C.	After 1 Hour at 62 C.
11..........................	320	320	0
12..........................	320	320	0
13..........................	5000	5000	0
14..........................	1280	1280	0
15..........................	640	640	0
Rabbit, after injections with X19..........................	10,000	10,000	10,000

Australia, the serums from which lost their agglutinins after heating to 65 C. for 30 minutes, but were still present after inactivation at 56 C.

The effect of heating has been tried with several serums of the series here described. Heating to 56 C. for one hour had no effect on the agglutinins but exposure to 62 to 64 C. for one hour resulted in total destruction. The serum of a rabbit injected with B. proteus X19 culture still retained its agglutinins after heating to 70 C. for one hour. Representative samples are given in table 2.

There seems to be conflicting evidence in the literature regarding the exact temperature at which the agglutinins for B. proteus X19 produced during an attack of typhus fever are destroyed, but it seems clear that, compared with the specific agglutinins produced in the rabbit by injections of proteus X19 cultures, the agglutinins produced in Brill's disease are relatively thermolabile. While they may not be destroyed at so low a temperature as those produced by old world typhus, they do not resist a temperature of 62 C. which has no effect on the specific agglutinins.

6 Deutsch. med. Wchnschr., 1917, 43, p. 1130.
7 Wien. klin. Wchnschr., 1917, 30, p. 1264.
8 München. med. Wchnschr., 1918, 65, p. 1377.
9 Ztschr. f. Hyg. u. Infektionskrankh., 1924, 103, p. 420.
10 M. & Sc. Arch. Adelaide Hosp.: Suppl. Annual Rep., 1923, 3, p. 20.

Weil and Breinl [11] have shown that agglutinins against X19 are produced in the rabbit by the injection of brain emulsions of guinea-pigs during the height of the febrile reaction, caused by typhus virus. The following experiments were made to determine the thermolability of the agglutinins produced in this manner with the virus of Brill's disease.

Rabbit 1.—Inoculated intraperitoneally Jan. 8 with 8 cc. of 1:20 suspension of brain of guinea-pig M-79 in salt solution (9th generation). Pigs of preceding generations of this strain were resistant to inoculation with old world typhus virus. Titer of serum at time of inoculation 1:4. Bled Jan. 22. Titer of serum 1:32.

Rabbit 2.—Inoculated intraperitoneally Jan. 6 with 6 cc. of 1:20 suspension in salt solution of brain of guinea-pig B-70 (4th generation). Titer of rabbit serum at time of inoculation was less than 1:4. Bled Jan. 20. Titer of serum 1:8.

Rabbit 3.—Inoculated intraperitoneally, Jan. 9, with 8 cc. of guinea-pig brain suspension (M-81) (7th generation). Titer of serum at time of inoculation 1:4. Bled Jan. 23. Titer of serum 1:32.

The effect of heat on the agglutinins thus produced in the serum of rabbits by the injection of guinea-pig passage virus is shown in table 3.

It is evident that the same relative thermolability exists for the agglutinins for B. proteus X19, produced by the virus of Brill's disease, in rabbit as in human serum.

TABLE 3

EFFECT OF HEAT ON AGGLUTININS FOR PROTEUS X19 IN RABBIT SERUM FOLLOWING INJECTIONS OF BRILL'S DISEASE VIRUS

Rabbit	Titers of Serums			
	Normal Less than	Immune	55 C. 1 Hour	62 C. 1 Hour
1......................................	1:4	1:32	1:32	1:8
2......................................	1:4	1:8	1:8	1:4
3......................................	1:4	1:32	1:32	1:4

DISCUSSION

Our first experience with Brill's disease in Alabama has been published.[12] Eleven cases, clinically identical with Brill's disease were described. All of these gave a positive Weil-Felix test in significant dilutions (1 : 80 to 1 : 1,280). In view of lack of evidence of the accepted vector of typhus (lice), a definite diagnosis of mild typhus or Brill's disease was, at that time, withheld.

Since that time further study of the disease by Maxcy has developed the following facts. Some 200 cases have been investigated in the past two years, all of which show the characteristic clinical picture of Brill's disease.[13] Guinea-pig inoculations have been made with the blood of a small series of these cases obtained during the height of the eruption.[14] The inoculated pigs showed the characteristic febrile reaction to typhus

[11] J. Infect. Dis., 1923, 33, p. 60.
[12] Am. J. Trop. Med., 1923, 3, p. 495.
[13] Maxcy, K. F.: Pub. Health Rep., 1926, 41, p. 1213.
[14] Ibid., to be published.

virus, and this reaction was successfully transmitted to other guinea-pigs by inoculations of blood and brain emulsions. Preliminary tests with representative guinea-pigs from different generations of these strains have showed that they are immune to inoculation with known (old world) typhus virus. Guinea-pigs which had recovered from inoculations with the old world virus likewise failed to respond to injections of the virus of Brill's disease. Although the epidemiology [15] of the disease, as it exists in southern United States, is at present obscure, the clinical course, the Weil-Felix reaction and the immunity tests in guinea-pigs, leave little doubt that it is mild typhus.

The results here described are based on our further experience with the Weil-Felix reaction. There are two essential criteria in judging the value of a test of this character. One is its specificity. The number of false positive results obtained in the absence of the condition under consideration must be low. The other is the frequency and uniformity with which the reaction is elicited by the specific infection. Our experience with the Weil-Felix reaction indicates that it meets both these requirements. In almost 1,000 macroscopic tests no false positive results were obtained. All of the cases with a positive reaction were found, upon investigation, to have the characteristic clinical course of mild typhus as described by Brill.[16] Only three cases failed to give a positive Weil-Felix reaction after the first week of the disease, indicating that the test is elicited in a high percentage of all cases. The microscopic test, with dried blood, using dilutions of approximately 1 : 40 and 1 : 80, as commonly employed in the Widal reaction, on the other hand, is less specific. False positive results are common, particularly when typhoid agglutinins are also present in the serum.

The differences between the agglutinins in Brill's disease and in virulent typhus seem to be merely relative, dealing with their thermolability, as shown by the fact that the agglutinins in Brill's disease are destroyed at 62 C. while the specific agglutinins against X19 resist 70 C. for one hour.

The experiments concerning the thermolability of the agglutinins, described in this paper, deal with three sources of origin, those produced in human serum by a natural attack of the disease, the serum of rabbits inoculated with guinea-pig passage virus and the serum of rabbits injected with cultures of B. proteus X19.

[15] Maxcy, K. F.: Pub. Health Rep., to be published.
[16] Am. J. M. Sc., 1910, 139, p. 484.

The latter are thermostable, our experiments confirming previous results that these agglutinins are not destroyed until a temperature of 70 to 75 C. is reached. This is in contrast to the thermolability of the agglutinins arising from a natural attack of typhus and those experimentally produced in rabbits by the injection of typhus virus. These, in our experience, never resisted a temperature higher than 64 C. for 1 hour, although our experience fails to confirm the observations already quoted that a temperature of 56 C. is sufficient for their destruction. It is, however, questionable if such small differences should be emphasized in the practical interpretation of the Weil-Felix reaction.

Brill's disease is not uncommon in the United States and seems to be widely distributed.[15] It is probable that the greater use of the Weil-Felix test by diagnostic laboratories and consideration of the Brill's disease syndrome in the differential diagnosis might help to clear up certain obscure febrile conditions.

SUMMARY

Results obtained with the macroscopic Weil-Felix test in over 100 cases of Brill's disease indicate that the reaction is elicited in about 95% of cases during the second week or later. It is usually negative during the first week, becoming increasingly stronger until convalescence. Tests including over 1,000 negative results demonstrate its specificity. Agglutination of B. proteus X19 in dilutions higher than 1 : 80 has not been obtained in other disease conditions.

The microscopic Weil-Felix test with dried blood has yielded false positive results, and, for this reason, is of doubtful value.

The agglutinins for B. proteus X19 in serums from patients with Brill's disease and also in serums from rabbits after injection of guinea-pig brain virus are relatively thermolabile; they are destroyed by heating the serum at 62 C. for 1 hour.

THE RELATION OF HYDROLYTIC DECOMPOSITION PRODUCTS OF PROTEINS TO BACTERIAL GROWTH

G. J. HUCKER and D. C. CARPENTER

From the New York State Agricultural Experiment Station, Geneva

The problem of securing an artificial medium in which bacteria will grow and produce all the changes which normally take place under natural conditions has confronted bacteriologists since the development of methods of pure culture study. It is well recognized that bacteria, in general, require food material similar to other living bodies but the exact form in which this food material, particularly the nitrogenous requirements, is most readily available has never been definitely determined. From our present knowledge of bacterial metabolism we know that the requirements of different groups of organisms vary over a wide latitude. The types most closely associated with animals and plants as parasites, are generally more particular in regard to their food sources when grown on artificial mediums than organisms isolated from saprophytic sources, such as soil, water, etc. The latter generally show a wider adaption in their ability to utilize various nutrient materials, probably due to the fact that the culture medium more closely resembles the conditions under which such organisms normally grow in nature. Naturally, more difficulty is experienced in securing a medium which approximates the natural conditions under which the more parasitic forms generally occur. This difficulty has been due largely to ignorance of the exact requirements of these more specialized parasites.

The carbon requirements of bacteria can be furnished in a form very similar to that found under natural conditions but the type of nitrogen most readily available is not so well known. For the particular organisms utilizing inorganic forms of nitrogen, such as many of the soil and water types, as well as a large majority of the yeasts, the most readily available nitrogen source is easily studied. But for those forms requiring organic nitrogen probably in the form of NH_2 or NH_3, the problem is much more difficult as the decomposition products of proteins appear to be the most readily available. Chemical methods have not been developed to properly separate these hydrolytic products in a pure form retaining their growth producing properties.

As the so-called "peptones" (commercial nitrogenous products which are prepared by splitting the protein molecule with enzymes) furnish the nitrogenous base of a large majority of mediums used in bacteriologic work, it was thought desirable to make a study of the availability of the various decomposition products of protein hydrolysis, as nitrogen sources for bacterial growth.

Previous to the isolation and study of bacteria in pure culture many early investigators working with the molds and yeasts used synthetic solutions containing various sugars and other carbohydrates as a source of energy, and ammonium salts, nitrates, and in some cases, amino acids, as asparagin, as sources of nitrogen. With the advent of pure culture study Pasteur, Cohn, and others, immediately adapted some of these solutions for the growth of bacteria. The earliest work, however, was with organisms isolated from beer, soil, etc., and this type of a synthetic medium furnished sufficient nutrient material for growth.

The first introduction of organic nitrogen into culture mediums other than through the use of urine, blood, and other body fluids, was probably by Naegeli[1] who used egg albumen, which he called "peptone." It was, however, some time later that partially digested proteins were found to be more available as nutrients and were placed on the market as "peptones" to be used as the nitrogenous base of culture mediums.

When it became evident that this so-called peptone was essential in the preparation of mediums in which to grow the more fastidious organisms, certain commercial brands appeared upon the market, notably "Witte," "Berne," "Fairchild," "Difco," etc. The process by which these peptones was prepared, and also the original protein used in the digestion was kept confidential by the various concerns, with the consequence that bacteriologists have had little information as to the definite composition of the nutrient nitrogenous materials used in mediums, and of the particular decomposition products most essential to produce the maximum growth.

The utilization of the hydrolytic protein decomposition products by bacteria has been investigated by many workers, and it is generally agreed that for the production of the greatest amount of growth, nitrogen must be available in the form of amino acids. Rettger, Berman and Sturges[2] have pointed out that free amino acids or some protein decomposition products are necessary in a medium before many organisms can initiate growth. Growth once started, however, certain organisms were then able through their enzymes to attack the more complex linkages. Later Koser and Rettger[3] have shown that pure amino acids furnished in a chemically definite medium will initiate growth of several test organisms used but many, particularly the cocci, failed to develop in the absence of protein decomposition products. Robinson and Rettger[4] found that "opsine," a biuret-free French product, served as an excellent source of nitrogen for even the most fastidious organisms.

The availability of proteins at different stages of enzymatic digestion, also, has been given considerable attention, particularly by English workers, and an

[1] Ernahrung der niederen Pilze durch Kohlenstoff und Stickstoffverbindungen. Untersuchungen über niedere Pilze. 1882, 1.
[2] J. Bact., 1916, 1, p. 15.
[3] J. Infect. Dis., 1919, 24, p. 301.
[4] J. M. Res., 1917, 31, p. 357.

outline of the earlier work is given by Hartley.[5] In general, it has been their conclusion that the more complete the digestion of the protein, the better the growth produced by various organisms which normally have special requirements. Cole and Lloyd[6] report, however, that although tryptic digests of casein and other proteins will produce a luxuriant growth of the meningococci, it generally fails to produce growth on the initial isolation in the absence of blood.

Recently Gordon and M'Leod[7] have carried on detailed experiments in which they have shown that certain amino acids when added to mediums appear to have a toxic action, while others are inert. A small group of amino acids and related compounds, however, have a decidedly beneficial action in the production of growth of pneumococci and meningocci. They point out that the addition of blood to tryptic digest enhances its usefulness by the blood acting as a protective agent against the toxic action of certain amino acids, as well as adding available nutrient materials to the medium. This explanation would account for the apparent variation in the action of different casein digests upon growth. To those who have had experience with the production of a tryptic casein digest medium it is a common observation to find one digest to act as a satisfactory medium for growth of streptococci and other related organisms, while another digest apparently prepared in the same manner will give entirely different results. The addition of serum to the unsatisfactory digest, however, generally greatly enhances its efficiency as a medium.

Beef Heart Muscle Digest as a Source of Nitrogen for Bacterial Growth.—Inasmuch as crude proteins of various nature are used as a basis for the commercial manufacture of "peptones," it was deemed advisable to observe the relative availability of the various hydrolytic products of digestion of some common proteins.

Finely ground heart muscle was subjected to digestion and samples removed at various intervals for test as to their ability to produce bacterial growth. The original native heart muscle was acidified, and after adding pepsin, was allowed to digest for eleven hours. At the end of this initial digestion, the digest was adjusted to about P_H 7.4 and trypsin added. The digest was then allowed to proceed for 36 hours. Samples were removed at regular intervals, heated to approximately 90 C., dried in vacuo, finely powdered, and retained for testing.

To test for the growth producing properties of the various stages of digestion, the minimum amount necessary to produce a visible growth was determined. Decreasing amounts of the dried samples were made to a given volume in physiologic salt solution, reaction adjusted to P_H 6.8-7.0, and after tubing and sterilizing in 20 mm. tubes were inoculated with a small loop of a suspension of the test organisms. The smallest percentage of peptone showing a visible clouding of the salt by the growth of the organism was recorded as the minimum amount necessary to produce growth.

Micrococcus aureus was adopted in the initial trials as a test organism and the minimum amounts of the various stages of digestion of beef

[5] J. Path. & Bact., 1922, 25, p. 479.
[6] Ibid., 1916, 21, p. 267.
[7] Ibid., 1926, 29, p. 13.

heart muscle necessary to product growth were determined (fig. 1). The tests for growth were repeated several times with the same comparable results. It appears that M. aureus, at least, requires for growth the complex linkages which are present in the first stages of digestion, as well as the cleavage products remaining at the end of the digestion, but the intermediate stages do not contain nitrogen in as available forms.

It can be noted that the peptic digest liberated between the sixth and eleventh hour either some toxic or other growth hindering substances, or nitrogen linkages which were not readily available for bacterial growth. This followed a period at the beginning of the digest in which the growth producing properties were practically unchanged. On the addition of the trypsin and consequent changes in the reaction, the efficiency of the digest remains practically constant with a rapid increase in the relative amount of growth producing substances present after 21 hours, reaching a maximum at 36 hours. Samples were not taken beyond this point.

Inasmuch as M. aureus is a facultative parasite and represents a special group of organisms, the minimum amounts of the digest at the various stages to produce growth of Serratia marcescens (Bacillus prodigiosus) were similarly determined. The resulting curve was similar in nearly every respect to that shown by Micrococcus aureus.

To secure further information, the growth producing properties of the digest was again tested using a Rawling's strain of the typhoid organism as a representative of the parasitic organisms. The results (fig. 1) are in some details different than those obtained in using either of the other test organisms, but, in general show a similar curve. These results with the typhoid organism are similar to those obtained using the species mentioned above in that during the digestion a period occurs in which the amount of the growth producing substances is reduced to a minimum, followed by a rapid increase as based upon the minimum amount necessary to produce growth.

The points at which the largest amounts of the digest were necessary to produce a visible growth of the typhoid organism occurred, however, over a longer period in the digestion than in the case of Micrococcus aureus. In the case of the typhoid organism, the point at which the largest amount of digest is necessary is at the end of the peptic digestion, similar to that noted for the other two test organisms, and on the addition of the trypsin and the change in reaction, the growth producing properties from the standpoint of the typhoid organism remained constant until the 29th hour in contrast to the 21st hour for Micrococcus aureus and Serratia marcescens, but at the end of the 36th hour when

the final sample was taken the amount of growth producing substances available for the typhoid organism had materially increased.

In the above cases, using either Micrococcus aureus or the typhoid organism, the addition of glucose to the test mediums as a source of energy had no apparent effect upon the curve. When the above titrations were repeated with the addition of glucose the same type of curve was obtained but in each case slightly less digest was required to produce growth.

Inasmuch as sufficient nitrogenous substance can be carried over with the inoculum to initiate growth, the titration tests were again repeated
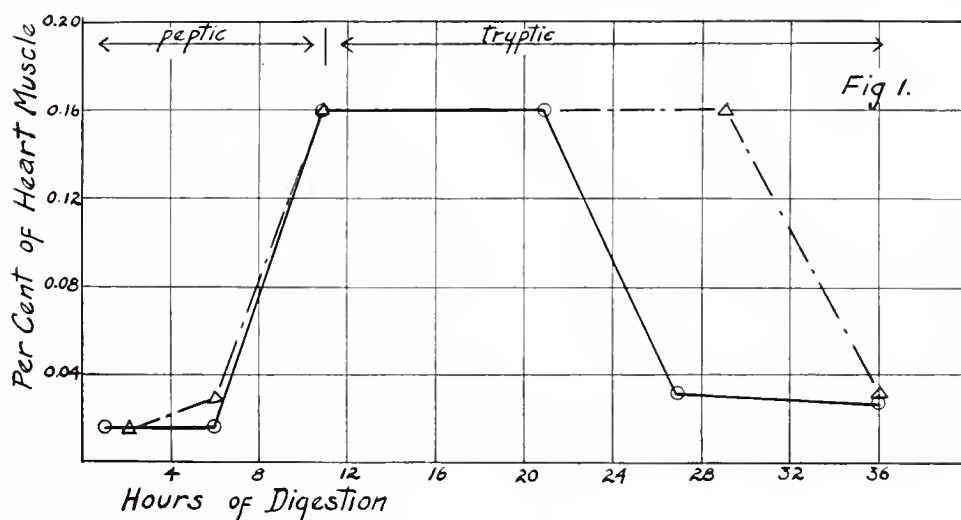
Fig. 1.—The minimum amount of digested beef heart muscle necessary to produce visible growth: Solid line (————): micrococcus aureus; and broken line (——.——): typhoid organism (Rawlings strain).

using flasks, instead of test tubes, containing 100 cc. of the medium. The inoculation was made with a small loop from a light salt suspension of the test organism. This method practically excluded the possibility of transferring nitrogenous material. Using this method the same type of curve was found in each instance.

Another factor which it was thought may have some effect upon the growth of the test organisms was the absence of many essential mineral salts when salt solution was used as the basis for the test medium. Repeated trials using a nitrogen free complete nutrient solution as a diluent in place of the physiologic salt solution gave similar results to the curves obtained when saline alone was used.

Nitrogen Distribution in Hydrolyzed Beef Heart Muscle.—The total nitrogen and amino nitrogen content of the dried samples of beef heart

muscle above mentioned which had been subjected to digestion by pepsin and also by trypsin for different periods of time were determined, in an effort to measure the completeness of the hydrolysis of the protein and to ascertain if any connection could be found between the growth curves and the amount of hydrolysis which had taken place in the protein.

Total nitrogen was determined by the usual Kjeldahl-Gunning method and amino nitrogen by the method of Van Slyke. All determinations were made in triplicate.

It is to be noted (table 1, and fig. 2) that as long as the solvent medium of the digest is of acid reaction (samples 1, 2, and 3) the total nitrogen remains practically constant as would be expected, and the amino nitrogen slowly increases due to the peptic digestion which is taking place. As soon as the solvent medium is made alkaline, however (samples 4-7), the total nitrogen rapidly decreases due, no doubt, to

TABLE 1

Total Nitrogen and Amino Nitrogen in Digested Beef Heart Muscle (Dried)

Sample	Enzyme	Hours of Digestion	Total N %	Amino N %	Amino N / Total N
1	Pepsin	2	12.74	3.39	0.266
2	Pepsin	5	12.71	3.58	0.282
3	Pepsin	11	12.69	3.68	0.290
4	Trypsin	17	12.32	4.98	0.404
4	Trypsin	21	11.85	5.94	0.501
6	Trypsin	27	11.11	5.51	0.496
7	Trypsin	36	9.94	5.07	0.510

the removal of ammonia during the process of drying the protein sample, subsequent to ammonia formation through the hydrolysis of amides.

The splitting action of trypsin appears to be energetic up to the point where one-half of the total nitrogen is transformed to amino nitrogen. Beyond this point trypsin appears to act only about as fast as amide nitrogen is converted into ammonia, maintaining practically a constant ratio between amino nitrogen and total nitrogen of 1:2 for at least 15 hours.

Sample 5 after 21 hours of digestion shows the maximum amino nitrogen content of the series, but reference to figure 1 shows that this amino nitrogen cannot be as available as that contained in sample 3 after eleven hours of digestion for the organisms used. On the basis of the experiments reported, the amino nitrogen content is little or no indication as to the desirability of a peptone sample for bacterial growth.

Raw Lean Beef Digest as a Source of Nitrogen for Bacterial Growth.—Raw lean beef was procured and freed as far as possible from ash and extraneous substances.

This material was submitted to tryptic digestion, and at intervals of two hours samples were removed and after heating quickly to about 70 C. to kill the action of the enzyme, was dried in vacuo. Samples representing the two, four, and six hour intervals were retained for tests for growth producing properties.

The determination of the minimum amount necessary to produce growth of the typhoid organism was determined as in the case of the digested heart muscle, the tests being made in flasks, using 100 cc. of medium and inoculating with only a small amount of a light suspension.

In general (fig. 3), the results of using digested beef were similar to those obtained using the digested heart muscle. The determinations
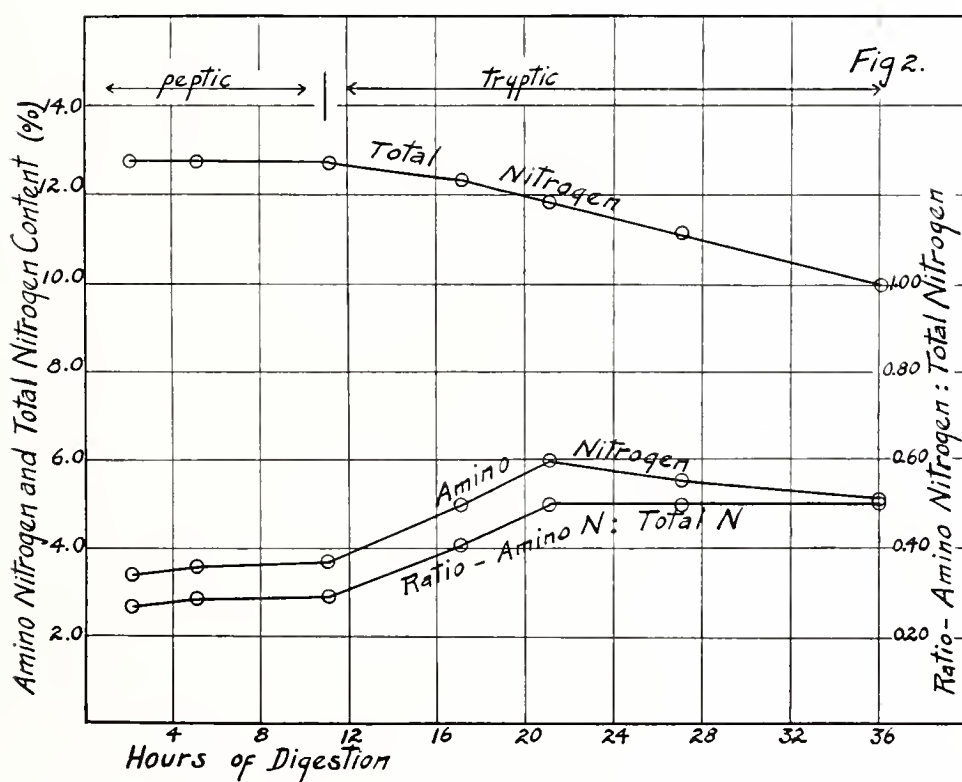
Fig. 2.—The amino nitrogen and total nitrogen content of digested beef heart muscle at various stages of digestion.

for the three intervals in the digestion were repeated several times and under numerous conditions, always with the same comparable results.

In the case of the digested lean beef, the digestion appears to have progressed much faster than in the case of the heart muscle. At the beginning of the digestion the amount of available growth producing substances quickly diminished, and at the end of four hours reached a point which required the largest amount of digest to produce a visible growth of the typhoid organism. Following this, however, the digest rapidly

became more efficient as a nutrient, and contained more available nutrient substances, and at the end of six hours had reached nearly a maximum in growth producing properties. This same digest when allowed to continue for two weeks required only .04% to produce growth. This amount is not materially smaller than the amount required at the end of six hours of digestion.

There are, of course, several points of difference in the two digests from the standpoint of their growth producing properties. The digestion
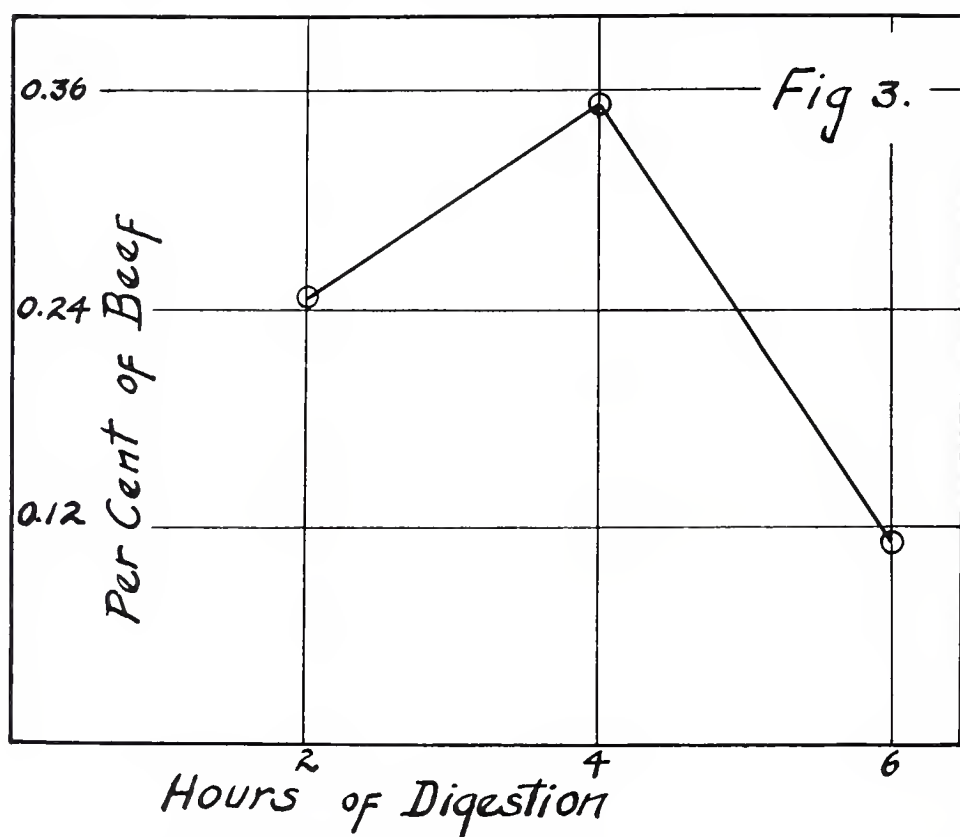
Fig. 3.—The minimum amount of digested (tryptic) raw lean beef necessary to produce visible growth of the typhoid organism (Rawlings strain).

of the heart muscle, both with pepsin and then with trypsin, proceeded slowly and showed a wide range in which the growth producing substances were at a minimum. The beef digestion with trypsin alone appeared to progress more rapidly, and the point at which the largest amount was required for growth was more quickly reached, and the digest rapidly developed more available nitrogenous material for bacterial growth.

Digest Mediums for Total Plate Counts of Milk.—The various fractions of the digestion as described above were tested further by using each as the only source of nitrogen in a number of comparative total plate counts of milk. In all, some 30 samples were plated at various times, and each time with a new set of mediums prepared from the digestion samples. The counts (figs. 4 and 5) show, in general, a close relationship to curve as obtained for the minimum amount of the digestion necessary to produce growth. Only six of the counts are plotted, but
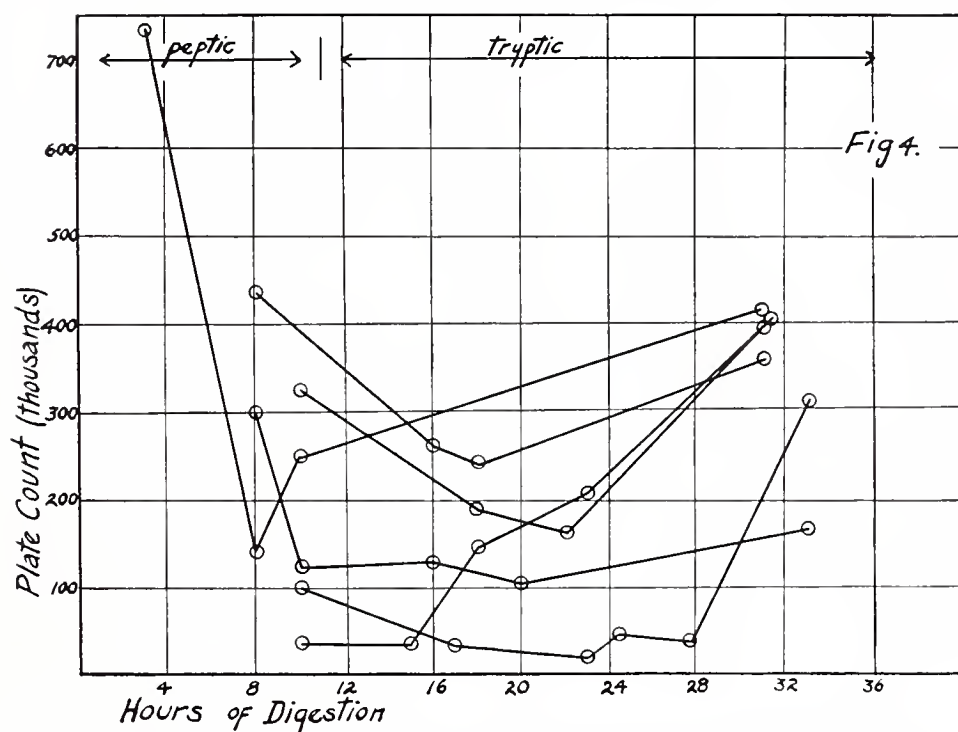
Fig. 4.—Plate counts of six samples of milk using digested beef heart muscle as the only source of nitrogen.

without exception all agreed in the comparative total counts with those which are shown.

In the case of the digested lean beef (fig. 5), it can readily be noted that the two hour and six hour samples gave the higher counts, while the four hour sample in no case gave as high a count. This observation agrees with the efficiency of the digest as determined by the titration method, as greater amounts of the four hour sample were required to produce a visible growth than with the other two samples. The variation in the plate count is quite striking as certain samples of milk varied from a count of 775,000 to 370,000 depending upon the stage of digestion of the peptone used. The plates were similarly handled and the same technic
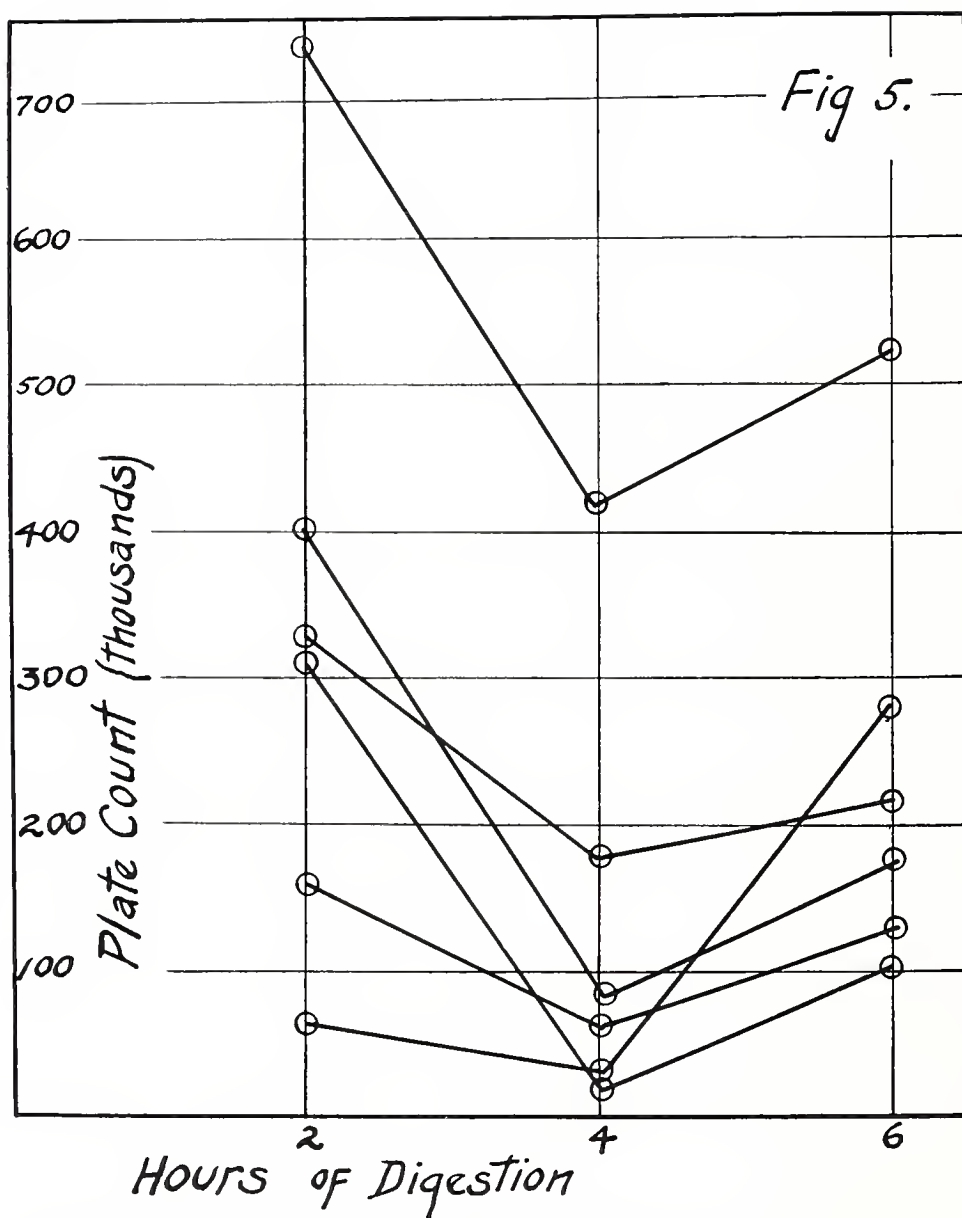
Fig. 5.—Plate counts of six samples of milk using digested (tryptic) raw lean beef as the only source of nitrogen.

followed in all cases so that other factors which may normally effect the count were eliminated.

In using the digested heart muscle as the only source of nitrogen in the medium used for the total count (fig. 4), the same general correlation between the counts and the titration or growth curve can be noted. The earlier stages of the digestion and the fractions obtained at the later part of the digestion gave the highest counts when the same sample of milk was plated using the various stages of the digested heart muscle as the source of nitrogen. The fractions obtained at the 10th and 22nd hour produced counts which were practically constant, while the later stages of the digestion gave the higher counts.

DISCUSSION

In digesting proteins in the preparation of nitrogenous bases for bacteriologic mediums, the important problems seems to be whether the variability in the available nitrogen for bacterial growth is due to the stage of the digestion or the type of the original protein being digested. From the foregoing results, it would appear that within certain limits the stage of digestion of the protein used in the manufacture of commercial "peptone" is an important factor. These data, however, deal only with two different but closely related types of protein, but the results are strikingly suggestive in pointing out the importance of the amount of digestion.

The work of Gordon and M'Leod [7] who found that certain amino acids, particularly tryptophan, are toxic, would indicate that digested proteins which have a low tryptophan content would yield very efficient peptones. This, however, is not the case, as gelatin, which is practically tryptophan free, when digested by the usual procedure followed in the manufacture of the Fairchild brand of commercial peptone, gave a very low comparative count from a number of milk samples. It contained, according to chemical analysis, a relatively large amount of total nitrogen and an exceptionally high percentage of nitrogen as peptone, viz., 12%, while the highest percentage found for certain other peptones was approximately 9%. The absence of tryptophan seemed to be the only definite factor which would account for the low counts secured when this special peptone was used as a nitrogen basis in mediums used for plating milk. In this case tryptophan appeared to be essential rather than toxic.

From these observations one would conclude that the stage of digestion is an important factor, providing that either a full complement of

amino acids are present in the original protein, or at least, that the essential ones are present in sufficient amount to have their characteristic effect upon growth when in available form.

A study of the above results, although showing the same type of curve, indicates, also, certain differences. The range through which the typhoid organism requires a large amount of the digest to produce a visible growth is much wider than that shown by Micrococcus aureus and Serratia marcescens. Although when grown on ordinary mediums the typhoid organism apparently grows very abundantly, it is evident that it is more sensitive in its food requirements and its selection of available nitrogen is more limited.

In the light of the above results, it is evident that another and important factor has entered into the matter of total plate counts. The variability of the counts as obtained in the sanitary control of milk and water supplies may be due, in part, to the various brands of commercial peptone found upon the market. In addition to being prepared from different original proteins, the stage of the final digestion differs greatly. The original "Witte peptone" contained a large amount of proteose, and was only slightly digested in comparison with certain other brands. "Fairchild's peptone," on the other hand, is more completely digested, while the only sample of "Berne peptone" available showed large amounts of proteose. "Bacto peptone" appears to be intermediate between Witte and Fairchild. When the range of these peptones is considered in the light of the results above, it is evident that the peptone may have a decided effect upon the numbers of colonies appearing upon the plates.

CONCLUSIONS

The amount of nitrogen available for bacterial growth shows considerable variation during the digestion process of proteins when determined by the minimum amount necessary to produce a visible growth of certain test organisms. There appears to be no relationship between the amount of free amino nitrogen present and the ability of partially digested proteins to produce bacterial growth.

There usually appears, following about the sixth to the eighth hour of the digestion, a period when relatively large amounts of the digest are required to promote growth. Earlier in the digestion and also after this period, the percentage of protein necessary for growth is much smaller.

Although the original protein may have some effect on the amount of available nitrogen for bacterial growth in the digest, the stage of the digestion is also an important factor.

FURTHER STUDIES ON THE STANDARDIZATION OF BACTERIAL VACCINES BY PHOTO-METRIC METHODS

ADELAIDE B. BAYLIS

From the Department of the Laboratories, New York Post-Graduate Medical School and Hospital

In a previous communication [1] on the standardization of typhoid vaccine, the reasons for investigation in this field and the technic of the method of photometric estimation were presented. In the present paper we purpose not to deal with these phases of the subject but to proceed at once to a consideration of the results obtained in further experience with the paratyphoid bacilli. The technic has been the same as that previously followed and continued experience with the procedure has made possible more consistent readings, serving further to emphasize the fact that estimation of bacterial vaccines by aid of the photomètre is both practicable and accurate.

The strains of typhoid and paratyphoid bacilli employed in these studies showed certain differences in form in microscopic examination. The individual organisms of Eberthella typhi appeared somewhat longer and more slender than those of Salmonella schottmülleri (emend), but the average volume of the individual organisms of the two species appeared equal. In comparison the individual cells of Salmonella paratyphi appeared distinctly larger on the average. The data of the photometric readings are in accord with these observations. The graphs presented for Eberthella typhi and Salmonella schottmülleri are remarkably similar while Salmonella paratyphi exhibits a more marked effect on the light in the same numerical concentrations.

Photometric readings would thus appear to be of value in demonstrating volume differences between organisms and also in detecting morphologic differences in the same strain which might arise as a result of alteration in mediums or other environmental influences.

The readings of the present study were made on ten bacterial suspensions prepared in each instance from the same strain of the respective organism. Each suspension was diluted so as to give the concentrations indicated in the second column of the table. In the upper part of each

Fig. 1 and 2.—Graphs of selected (———), maximum (———), and minimum (———) readings of absorption and diffusion (with indicated dilutions) of Salmonella paratyphi.

Figs. 3 and 4.—Graphs of selected (———), maximum (———) and minimum (———) readings of absorption and diffusion with indicated dilutions of Salmonella schottmülleri.

SALMONELLA PARATYPHI: ABSORPTION

Actual Readings

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	1.48 0/A	1.44 0/A	1.50 0/A	1.50 0/A	1.45 0/A	1.48 0/A	1.49 0/A	1.50 0/A	1.44 0/A	1.48 0/A	1.47 0/A
2	1000	1.64 0/A	1.62 0/A	1.68 0/A	1.62 0/A	1.62 0/A	1.65 0/A	1.66 0/A	1.65 0/A	1.62 0/A	1.65 0/A	1.65 0/A
3	1500	1.86 0/A	1.85 0/A	1.86 0/A	1.86 0/A	1.85 0/A	1.86 0/A	1.87 0/A	1.86 0/A	1.84 0/A	1.85 0/A	1.87 0/A
4	2000	2.03 0/A	2.02 0/A	2.06 0/A	2.01 0/A	2.00 0/A	2.06 0/A	2.04 0/A	2.05 0/A	2.02 0/A	2.05 0/A	2.02 0/A
5	2500	2.22 0/A	2.21 0/A	2.21 0/A	2.20 0/A	2.20 0/A	2.22 0/A	2.23 0/A	2.21 0/A	2.22 0/A	2.22 0/A	2.23 0/A
6	3000	2.39 0/0	2.40 0/0	2.38 0/A	2.40 0/A	2.39 0/A	2.39 0/A	2.39 0/A	2.40 0/A	2.39 0/A	2.40 0/A	2.39 0/A
7	3500	1.43 0/0	1.42 0/0	1.42 0/0	1.43 0/0	1.43 0/0	1.43 0/0	1.43 0/0	1.42 0/0	1.42 0/0	1.43 0/0	1.42 0/0
8	4000	1.68 0/0	1.70 0/0	1.69 0/0	1.67 0/0	1.67 0/0	1.67 0/0	1.68 0/0	1.67 0/0	1.69 0/0	1.67 0/0	1.67 0/0
9	4500	1.86 0/0	1.87 0/0	1.85 0/0	1.85 0/0	1.85 0/0	1.85 0/0	1.85 0/0	1.87 0/0	1.87 0/0	1.89 0/0	1.85 0/0
10	5000	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.05 0/0	2.06 0/0	2.05 0/0

Calculated Absorption (0/0)

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	0.34	0.30	0.36	0.36	0.31	0.34	0.35	0.36	0.30	0.34	0.35
2	1000	0.50	0.48	0.54	0.48	0.48	0.51	0.52	0.52	0.48	0.51	0.51
3	1500	0.72	0.71	0.72	0.72	0.71	0.72	0.73	0.72	0.70	0.71	0.73
4	2000	0.89	0.88	0.92	0.87	0.86	0.92	0.90	0.91	0.88	0.91	0.88
5	2500	1.08	1.07	1.07	1.06	1.06	1.08	1.09	1.07	1.08	1.08	1.09
6	3000	1.25	1.26	1.24	1.26	1.25	1.25	1.25	1.26	1.25	1.26	1.25
7	3500	1.43	1.42	1.42	1.43	1.43	1.43	1.43	1.42	1.42	1.43	1.42
8	4000	1.68	1.70	1.69	1.67	1.67	1.67	1.68	1.67	1.69	1.67	1.67
9	4500	1.86	1.87	1.85	1.85	1.85	1.85	1.85	1.87	1.87	1.89	1.85
10	5000	2.05	2.05	2.05	2.05	2.05	2.05	2.05	2.05	2.05	2.06	2.05

TABLE 2

SALMONELLA PARATYPHI: DIFFUSION

Actual Readings, B/0

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	2.25	2.33	2.22	2.22	2.33	2.22	2.23	2.22	2.33	2.23	2.23
2	1000	2.07	2.10	2.06	2.07	2.07	2.06	2.07	2.05	2.10	2.06	2.07
3	1500	1.90	1.90	1.89	1.90	1.91	1.90	1.90	1.88	1.91	1.86	1.91
4	2000	1.79	1.79	1.79	1.79	1.80	1.79	1.78	1.79	1.79	1.76	1.79
5	2500	1.71	1.72	1.69	1.71	1.71	1.70	1.71	1.71	1.72	1.69	1.70
6	3000	1.61	1.62	1.63	1.61	1.62	1.61	1.61	1.61	1.63	1.60	1.62
7	3500	1.52	1.52	1.53	1.53	1.52	1.53	1.52	1.52	1.53	1.52	1.51
8	4000	1.46	1.46	1.45	1.47	1.46	1.47	1.46	1.48	1.46	1.46	1.46
9	4500	1.42	1.42	1.41	1.41	1.42	1.43	1.41	1.42	1.43	1.42	1.42
10	5000	1.38	1.37	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38

Calculated Diffusion

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	5.1625	5.2497	5.1298	5.1298	5.2497	5.1298	5.1407	5.1298	5.2497	5.1407	5.1407
2	1000	4.9663	4.9990	4.9554	4.9663	4.9663	4.9554	4.9663	4.9145	4.9990	4.9554	4.9663
3	1500	4.7810	4.7810	4.7701	4.7810	4.7919	4.7810	4.7810	4.7592	4.7919	4.7374	4.7919
4	2000	4.6611	4.6611	4.6611	4.6611	4.6720	4.6611	4.6602	4.6611	4.6611	4.6284	4.6611
5	2500	4.5739	4.5848	4.5521	4.5739	4.5739	4.5630	4.5739	4.5739	4.5818	4.5521	4.5630
6	3000	4.4649	4.4758	4.4867	4.4649	4.4758	4.4649	4.4649	4.4649	4.4867	4.4540	4.4758
7	3500	4.3668	4.3668	4.3777	4.3777	4.3668	4.3668	4.3668	4.3668	4.3777	4.3668	4.3559
8	4000	4.3014	4.3014	4.2005	4.3123	4.3014	4.3123	4.3014	4.3232	4.3014	4.3014	4.3014
9	4500	4.2578	4.2578	4.2469	4.2569	4.2578	4.2469	4.2469	4.2578	4.2687	4.2578	4.2578
10	5000	4.2142	4.2033	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142

TABLE 3

SALMONELLA SCHOTTMÜLLERI: ABSORPTION

Actual Readings

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	1.41 0/A	1.41 0/A	1.40 0/A	1.41 0/A	1.40 0/A	1.41 0/A	1.41 0/A	1.41 0/A	1.41 0/A	1.41 0/A	1.41 0/A
2	1000	1.58 0/A	1.57 0/A	1.56 0/A	1.57 0/A	1.57 0/A	1.58 0/A	1.58 0/A	1.58 0/A	1.58 0/A	1.58 0/A	1.58 0/A
3	1500	1.75 0/A	1.75 0/A	1.75 0/A	1.74 0/A	1.74 0/A	1.75 0/A	1.76 0/A	1.75 0/A	1.75 0/A	1.75 0/A	1.75 0/A
4	2000	1.94 0/A	1.94 0/A	1.94 0/A	1.94 0/A	1.93 0/A	1.94 0/A	1.94 0/A	1.94 0/A	1.95 0/A	1.95 0/A	1.95 0/A
5	2500	2.08 0/A	2.08 0/A	2.08 0/A	2.05 0/A	2.06 0/A	2.09 0/A	2.08 0/A	2.08 0/A	2.09 0/A	2.08 0/A	2.08 0/A
6	3000	2.18 0/A	2.18 0/A	2.18 0/A	2.18 0/A	2.16 0/A	2.90 0/A	2.19 0/A	2.19 0/A	2.19 0/A	2.18 0/A	2.18 0/A
7	3500	2.33 0/A	2.32 0/A	2.31 0/A	2.32 0/A	2.31 0/A	2.33 0/A	2.32 0/A	2.34 0/A	2.34 0/A	2.33 0/A	2.33 0/A
8	4000	1.40 0/0	1.40 0/0	1.40 0/0	1.38 0/0	1.40 0/0	1.40 0/0	1.41 0/0	1.41 0/0	1.40 0/0	1.41 0/0	1.40 0/0
9	4500	1.67 0/0	1.67 0/0	1.68 0/0	1.67 0/0	1.67 0/0	1.67 0/0	1.68 0/0	1.68 0/0	1.67 0/0	1.67 0/0	1.67 0/0
10	5000	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0	1.79 0/0

Calculated Absorption (0/0)

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	0.27	0.27	0.26	0.27	0.26	0.27	0.27	0.27	0.27	0.27	0.27
2	1000	0.34	0.33	0.32	0.33	0.33	0.34	0.34	0.34	0.34	0.34	0.34
3	1500	0.61	0.61	0.61	0.60	0.60	0.61	0.62	0.61	0.61	0.61	0.61
4	2000	0.80	0.80	0.80	0.80	0.79	0.80	0.80	0.80	0.81	0.81	0.81
5	2500	0.94	0.94	0.94	0.91	0.92	0.95	0.94	0.94	0.95	0.94	0.94
6	3000	1.04	1.04	1.04	1.04	1.02	1.06	1.05	1.05	1.05	1.04	1.04
7	3500	1.19	1.18	1.17	1.18	1.17	1.19	1.18	1.20	1.20	1.19	1.19
8	4000	1.40	1.40	1.40	1.38	1.40	1.40	1.41	1.41	1.40	1.41	1.40
9	4500	1.67	1.67	1.68	1.67	1.67	1.67	1.68	1.68	1.67	1.67	1.67
10	5000	1.79	1.79	1.79	1.79	1.79	1.79	1.79	1.79	1.79	1.79	1.79

TABLE 4

SALMONELLA SCHOTTMÜLLERI: DIFFUSION

Actual Readings, B/0

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	2.38	2.38	2.38	2.38	2.38	2.38	2.38	2.38	2.38	2.38	2.38
2	1000	2.13	2.13	2.13	2.13	2.13	2.13	2.13	2.12	2.13	2.13	2.13
3	1500	1.89	1.89	1.89	1.90	1.90	1.80	1.90	1.89	1.90	1.88	1.89
4	2000	1.83	1.83	1.84	1.83	1.83	1.84	1.83	1.83	1.83	1.83	1.83
5	2500	1.78	1.78	1.78	1.78	1.79	1.78	1.79	1.79	1.78	1.78	1.78
6	3000	1.68	1.68	1.68	1.69	1.70	1.69	1.68	1.68	1.69	1.68	1.68
7	3500	1.58	1.58	1.59	1.58	1.60	1.58	1.59	1.59	1.58	1.58	1.58
8	4000	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.57	1.56	1.56	1.56
9	4500	1.51	1.51	1.51	1.51	1.51	1.52	1.51	1.51	1.51	1.51	1.51
10	5000	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48

Calculated Diffusion

Tubes	Dilutions, Millions per Cc.	Selected Reading	1	2	3	4	5	6	7	8	9	10
1	500	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042	5.3042
2	1000	5.0317	5.0317	5.0317	5.0317	5.0317	5.0317	5.0317	5.0208	5.0317	5.0317	5.0317
3	1500	4.7701	4.7701	4.7701	4.7810	4.7810	4.7810	4.7810	4.7701	4.7810	4.7592	4.7701
4	2000	4.7047	4.7047	4.7156	4.7047	4.7047	4.7156	4.7047	4.7047	4.7047	4.7047	4.7047
5	2500	4.6502	4.6502	4.6502	4.6502	4.6611	4.6502	4.6611	4.6611	4.6502	4.6502	4.6502
6	3000	4.5412	4.5412	4.5412	4.5621	4.5630	4.5521	4.5412	4.5412	4.5521	4.5412	4.5412
7	3500	4.4322	4.4322	4.4431	4.4322	4.4540	4.4322	4.4431	4.4431	4.4322	4.4322	4.4322
8	4000	4.4104	4.4104	4.4104	4.4104	4.4104	4.4104	4.4104	4.4213	4.4104	4.4104	4.110'
9	4500	4.3559	4.3559	4.3559	4.3559	4.3559	4.3668	4.3559	4.3559	4.3559	4.3559	4.–
10	5000	4.3232	4.3232	4.3232	4.3232	4.3232	4.3232	4.3232	4.3232	4.3232	4.3232	4.32

table are recorded the actual photometric readings with the surcharge employed in each instance. In the third column the selected mean figures are recorded, these representing the average of the ten individual readings except where a certain reading has been in predominance and on this account chosen as the true value. The selected mean readings, and the maximum and minimum observations are shown in the graphs.

SUMMARY

Further experience with the photometric estimation of bacterial vaccines confirms the previous statement that this method offers precise accuracy combined with economy of time.

Observations on the absorption and diffusion produced by standardized suspensions of the paratyphoid bacilli are here recorded.

Photometric standardization of bacterial vaccines offers a check on variation in the average size of organisms in different cultures.

This data together with the similar data concerning the typhoid bacillus presented in the previous communication afford criteria for the photometric standardization of the components of the typhoid-paratyphoid vaccine.

THE INFLUENCE OF PURULENT INFECTION ON THE DEVELOPMENT OF EXPERIMENTAL SCURVY

Henry L. Jaffe

From the Laboratory Division of the Hospital for Joint Diseases, New York City

The relation of induced infection to experimental scurvy has been studied with reference to tuberculosis, and Höjer[1] found that this disease had no marked influence on the development and the extent of the scorbutic lesions in the different organs. In the course of an investigation of the regeneration of bone in scorbutic guinea-pigs, one of our animals developed an acute suppurative staphylococcus osteomyelitis. This animal was on a scurvy diet for 40 days, but developed no clinical signs of scurvy and at necropsy showed none of the cardinal lesions such as hemorrhages, beading at the costochondral junctions, and fractures. The abdominal fat was well preserved, the liver was large and fatty, and there was a splenomegaly of chronic infection. The animal weighed 322 grams when placed on the diet and 353 grams when killed. Its cage mates, which had been submitted to the same procedures, but which did not become infected developed severe acute scurvy after three weeks, and died with typical lesions within six weeks after the beginning of the experiment. This unusual result led me to gather more data on the possible relation between infection and scurvy.

Diet: This consisted of a soy bean flour base as used by Cohen and Mendel[2] and recently modified by Wolbach and Howe.[3] The formula is as follows: soy bean flour, 50 parts; rolled oats, 29 parts; dried milk powder, 10 parts; brewer's yeast, 4 parts; butter, 5 parts; calcium carbonate, 1 part; and sodium chloride, 1 part. The soy bean flour, oats, and milk were mixed in these proportions and autoclaved at 15 pounds pressure for 45 minutes. The salts were then added. An adequate amount of this mixture, to which fresh yeast and butter were added, was fed daily. In addition the animals were given distilled water ad libitum and hay was given for roughage. This diet is deficient only in the antiscorbutic vitamin.

The Effect of the Scorbutic Diet on Normal Guinea-Pigs.—Twelve guinea-pigs weighing from 200 to 370 grams were fed this diet. Between the 13th and 18th day all manifested signs of scurvy such as

[1] Acta paediat., 1924, suppl. 3, p. 8.
[2] J. Biol. Chem., 1918, 35, p. 427.
[3] Wolbach, S. B., and Howe, P. R.: Arch. Path., 1926, 1, p. 1.

swollen and tender wrists, weakness, tender hind legs, and loosening of the teeth. Seven succumbed on or before the 30th day and by the 42nd day all had either died of scurvy or had been killed because they were moribund. A few animals were killed for necropsy between the 14th and 18th days, and on histological examination already showed well developed scorbutic changes in the costochondral junctions. The ribs best demonstrate the typical scorbutic changes, which have been described recently by Aschoff and Koch,[4] Hess, Höjer,[1] and Wolbach and Howe.[5]
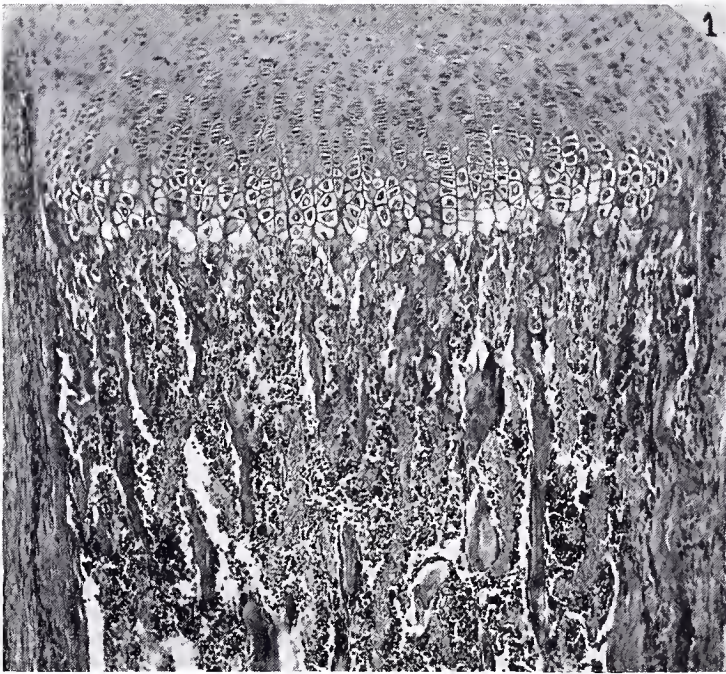
Fig. 1.—Costochondral junction of a normal guinea-pig, 47 days old.

They consist of cessation of new bone formation with diminution in the number of osteoblasts in the preparatory calcification zone, rarifaction of the existing cortex and spongiosa, irregularities and absorption and disappearance of the cartilage columns, and the formation of a zone of fragmented bone just distal to the costochondral junction, known as the Trümmerfeld zone. Fractures and hemorrhages frequently occur here. The marrow spaces of the shaft adjacent to the cartilage or Trümmerfeld zone, become filled with a loosely constructed fibrillar tissue on a

[4] Skorbut, eine Pathologisch-Anatomische Studie, 1919.
[5] Scurvy, Past and Present, 1920.

gelatinous appearing background ("Gerüstmark"). All our normal guinea-pigs fed on this diet for two weeks or more showed these changes, the severity varying with the length of time on the diet.

The Course of Scurvy in Infected Animals.—Eleven animals weighing between 198 and 428 grams were placed on the scorbutic diet and at the same time infected with 18 hour broth cultures of staphylococcus pyogenes albus by injecting a few minims of the culture into the marrow cavity of the tibia, muscles of the leg, or into an incised skin wound. In all, three injections were given at two or three day intervals. Six
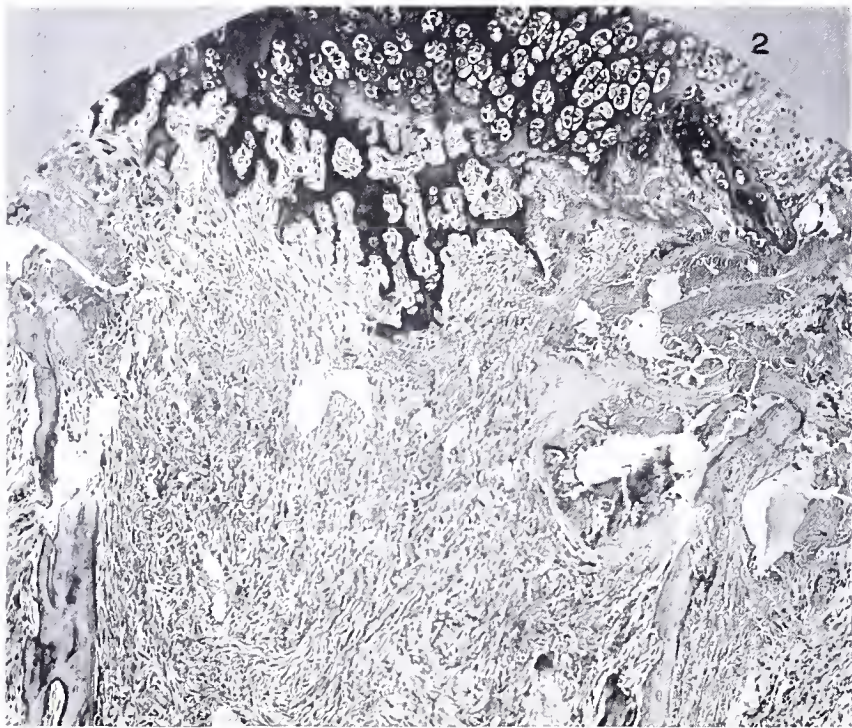
Fig. 2.—Costochondral junction of a guinea-pig 95 days old, on scurvy diet for 39 days, showing irregularity of the cartilage columns, Gerüstmark, Trümmerfeld zone, and fractures.

animals developed severe local infections with or without sepsis and when necropsied between the 19th and 40th days showed none of the classical changes of scurvy in either gross or histologic examination. The bones were somewhat brittle, due as shown on histologic examination to an osteoporosis. The livers were large and yellow and showed extensive fatty replacement of the liver cells, numerous areas of focal necrosis, and moderate leukocytic and lymphocytic infiltration in the peri-

portal spaces. The spleens were also large and pulpy, with markedly congested sinuses and prominent Malpighian corpuscles.

The remaining five animals died of scurvy between the 21st and 30th days. In none of these cases did the inoculations result in severe local infections, and the necropsies failed to reveal evidences of general infection or prolonged toxic absorption in the internal organs.

In this experiment severe infection prevented the appearance of scurvy in six of eleven guinea-pigs on a scurvy diet from 19 to 40 days. Normal animals on this diet developed scurvy after two weeks.
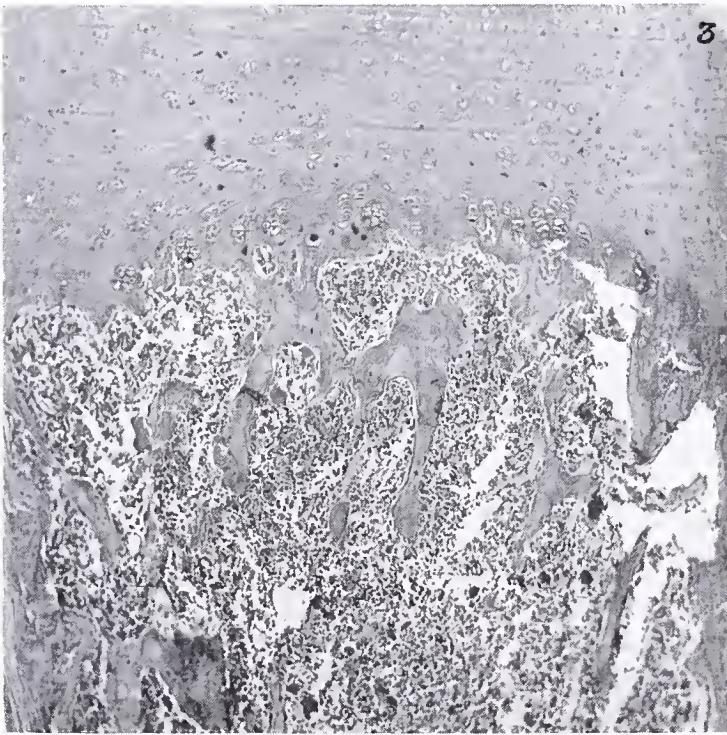
Fig. 3.—Costochondral junction of an infected guinea-pig 106 days old, on scurvy diet 40 days, showing atrophy and osteoporosis, but no scurvy.

The Effect of Infection on the Course of Scurvy Already Developed.—Six guinea-pigs weighing between 298 and 355 grams were placed on the scorbutic diet and ten days later, when signs of scurvy were first appearing, were injected with broth cultures of Staphylococcus albus as described before. In these animals death followed shortly after the development of the infections, three dying on the 7th day and the other three on the 12th day after the first injection.

The Influence of Staphylococcus Vaccine on the Course of Scurvy.—
Four guinea-pigs each weighing about 250 grams were given four intra-
peritoneal injections of standard Staphylococcus albus and aureus vac-
cine containing one billion bacteria per cc. The inoculations began on
the day the animals were placed on a scurvy diet, extended over a period
of 11 days, and were graded from 0.5 to 2.5 cc. By the 15th day these
animals had developed definite clinical signs of scurvy. Vaccine in the
amounts we injected had no preventive effect on the disease.

DISCUSSION

All authors studying the pathologic anatomy of human or experi-
mental scurvy emphasize the changes at the costochondral junctions as
best indicating the progress and extent of the disease. I studied the
costochondral junctions of our guinea-pigs and have interpreted the
absence of "Gerüstmark," Trümmerfeld zone, fractures, and hemor-
rhages as indications of the absence of scurvy. Some degrees of osteo-
porosis developed in the infected guinea-pigs. This I interpreted as due
to inanition.

I cannot explain why the onset and development of scurvy is delayed
for a long time or entirely prevented in guinea-pigs on a scorbutic
regime in the presence of a severe local staphylococcus infection, while
noninfected guinea-pigs on the same diet develop clinical and anatomical
evidences of scurvy regularly at about the 15th day. In conformity with
the vast experience of human scurvy our findings show that the course
of this disease is much more rapid if an infection is superimposed upon
existing scurvy.

The antiscorbutic power of the infection is not due to antibody for-
mation, for in our studies the course of scurvy was in no way influenced
by vaccination. The possibility that vitamin C was produced by bacte-
rial growth in the muscles must be considered. Although the production
of vitamin C by bacterial growth has not been demonstrated, the pro-
duction of vitamin A and B in this way has been proved.[6] Another
possibility is that protective substances were developed in the liver as the
result of toxic absorption.

That infection does not cause scurvy has been proved beyond a
doubt, but that a severe purulent infection present before the appearance
of active scurvy may delay or prevent the development of this disease in
guinea-pigs is suggested by these experiments.

[6] Burrows, M. T., and Jorstad, L. H.: Am. J. Physiol., 1926, 77, pp. 24, 37.

CONCLUSIONS

Six of eleven infected guinea-pigs on a scurvy diet developed severe purulent infections and did not develop scurvy within 19 to 40 days. Uninfected guinea-pigs on the same diet developed scurvy in 13 to 18 days.

The injection of Staphylococcus albus into animals with early signs of scurvy caused a rapid fatal termination. The course of scurvy was not influenced by the injection of standard staphylococcus vaccine.

These studies suggest the possibility that antiscorbutic substances may be developed by bacterial growth in the tissues or as a result of the action of absorbed toxins on the internal organs.

LEPTOSPIRA BIFLEXA

(WOLBACH AND BINGER, 1914. EMEND. NOGUCHI, 1918)

ONE PLATE

VLADIMIR T. DIMITROFF

From the Department of Pathology, Harvard University Medical School, Boston

Free living spiral microorganisms identical in morphology with the cause of infectious jaundice (Leptospira icterohemorrhagiae, Inado and Ido 1915, Emend, Noguchi 1917) (Spirochaeta icterogenes, Uhlenhuth and Fromme [1] 1915) have been found in several parts of the world. The resemblance of these free living leptospiras to Noguchi's Leptospira icteroides is equally exact.

Wolbach and Binger,[2] in 1913 (antedating the discovery of Leptospira icterohemorrhagiae) observed for the first time a free living leptospira. The organism which they named Spirochaeta biflexa was found in the water of Jamaica Pond, Boston, and was isolated by filtration through a Berkefeld "V" filter. It was described as very delicate spiral five to seven microns long with extreme closeness of the turns and curved or flexed tapered extremities. The amplitude or width of the spirals was given as 0.2 to 0.25 microns, and 22 to 32 spirals per organism. They also described the motion and the appearances of the organism in motion now familiar to workers with the Leptospira.* In recent years Leptospiras have been found in surface waters in various parts of the world and many attempts have been made to identify them with or to differentiate them from the pathogenic Leptospira icterohemorrhagiae. This paper presents the results of experiments with leptospiras found in fresh water from a number of cities in several Eastern States. They have been isolated in pure culture and their effect on guinea-pigs and mice has been tested.

Bach [3] cultivated spirochetes from the drinking water in Bonn. One species morphologically resembled Spirochaeta (Leptospira) pseudo-

[1] Berlin. klin. Wchnschr., 1916, p. 269.

[2] J. M. Research, 1914, 30, pp. 9, 23.

[3] Deutsch. med. Wchnschr., 1921, 47, p. 1080.

* In acceptance of Noguchi's nomenclature, microorganisms having the above characteristics will be referred to as Leptospira in this paper. Uhlenhuth and Zeulzer, however, do not accept the validity of Noguchi's criteria for creating a new genus.

icterogenes of Uhlenhuth and Zeulzer.[4] Noguchi[5] found leptospiras in water samples from Woods Hole, Massachusetts, from Shandaken and from other sections in New York State. They were present in fresh, stagnant and salt water but were not pathogenic for guinea-pigs and rats. Angerer[6] succeeded in filtering a strain of water leptospira and apparently cultivated it in pure cultures. Many other reports deal with the question of the association with or differentiation of ordinary water leptospira from that of infectious jaundice.

Uhlenhuth and Zeulzer[4] give data showing that Spirochaeta pseudo-icterogenes (water leptospira) is widely distributed in waters of various qualities. They have been overlooked because dark field illumination was not used for their detection. Zeulzer[7] found Spirochaeta icterogenes in the tap water of Berlin. In the course of time by successive passages these spirochetes became pathogenic for guinea-pigs and produced typical jaundice. Hindle[8] found leptospiras in the tap water of London, in the Thames River, in rain water, in the slime on the laboratory faucet and in other places. His animal experiments suggest, but do not clearly demonstrate their pathogenicity for guinea-pigs.

Manine, Cristau, and Plazy[9] reported a number of cases of Weil's disease among the French troops at L'Orient during the World War. They felt that water spirochetes are not identical with those reported among the soldiers at the front, yet the organisms produced similar symptoms in guinea-pigs. Uhlenhuth and Fromme[10] identified Inado's Spirochaeta icterhemorrhagiae in cases of Weil's disease among the German troops on the Western front. They, however, named this organism Spirochaeta icterogenes. Stakes and Ryle[11] were the first to report Spirochaeta icterohemorrhagiae in jaundice patients among the British troops in France. Bonini[12] reported an epidemic of Weil's disease among the soldiers on the Italian front in the region of Izonzo.

Several reports have associated epidemics of Weil's disease with bathing in polluted water. Reiter[13] definitely associated the occurrence of the disease in the German army, with bathing in certain pools in

[4] Centrlbl. f. Bakteriol., I, O., 1921, 85, p. 141.
[5] N. Y. State M. J., 1922, 22, p. 426.
[6] Arch. f. Hyg., 1924, 92, p. 325.
[7] Centralbl. f. Bakteriol., I, O., 1923, 89, p. 171.
[8] Brit. M. J., 1925, 2, p. 57.
[9] Compt. rend. Soc. de biol., 1917, 80, pp. 531, 574.
[10] Deutsch. med. Wchnschr., 1917, 43, p. 1553.
[11] Lancet, 1917, 192, p. 142.
[12] Gazz. d. Osped. e del. Clin., 1917, 38, p. 691.
[13] Ztschr. f. Med., 1919, 88, p. 495.

Magdeburg and Brunswick. Manson-Bahr, Wenyon, and Brown [14] reported a case of severe spirochetal jaundice in a sailor who had fallen into the Thames River near Grove Sand. The symptoms appeared three days after the incident. Inoculation of guinea-pigs with the urine and blood from this patient produced typical jaundice that proved fatal to the animals. Schoerer [15] reported a case of Weil's disease in a laborer who had fallen into a sewer 14 days before the onset of the symptoms. The disease was produced in guinea-pigs by inoculating them with the patient's blood. Etchegoin [16] reported several cases of infectious jaundice among people who had bathed in a certain swimming pool in France. He found the spirochetes in these pools and produced the disease in guinea-pigs. He further pointed out that these leptospiras were agglutinated by the patient's serum in dilutions of 1 : 2000. Koerner [17] by closing the swimming pool in Magdeburg succeeded in checking an epidemic among the police cadets.

Considerable work has been done to determine the method of transmission of infectious jaundice. The Japanese workers, Inado, Ido, Holi, Kaneki and Ito [18] announced that Spirochaeta icterohemorrhagiae is the etiologic agent of the disease. They demonstrated the spirochaetes in a patient's blood up to the thirteenth day of the illness and the blood was infectious for guinea-pigs for the same duration. They pointed out that the disease occurred most frequently among men working in wet mines. They suggested that infection takes place through the skin. Uhlenhuth and Kuhn [19] succeeded in transmitting the disease to guinea-pigs through the bite of the stable fly. Out of nine attempts, four were certain, three doubtful, and two negative. Foulerton [20] advanced evidence to show that infection among the miners in England takes place by eating food or drinking water contaminated with the spirochetes. Toyama [21] noticed that Weil's disease occurred among the workers in the paddy fields of Japan. His experiments with potassium cyanide which mixed with fertilizer and spread on the rice fields reduced the morbidity down to almost nothing; while the incidence in workers in the untreated fields continued as usual. Buchanan [22] found leptospiras

[14] Lancet, 1922, 22, p. 1056.
[15] Med. Klinik, 1922, 17, p. 533.
[16] Compt. rend. Soc. de biol., 1924, 91, p. 1005.
[17] Deutsch. med. Wchnschr., 1925, 51, p. 772.
[18] J. Exper. Med., 1916, 23, p. 37.
[19] Ztschr. f. Hyg. u. Infektionskr., 1917, 84, p. 517.
[20] J. Path. & Bact., 1919-1920, 23, p. 78.
[21] Trop. Med. Bull., 1924, 21, p. 275.
[22] Brit. M. J., 1924, 2, p. 990.

in the thin layers of slime deposited on the roofs of damp mines. Injections of 4 cc. of this slime intraperitoneally into guinea-pigs proved fatal in thirteen days. The slime from the old parts of the mines was most infectious, and the workers in those sections suffered more frequently from the disease. Contact with the slime and its drippings was supposed to be the source of infection. Gulland and Buchanan [23] concluded that infection of miners in England might be due to contamination of food with slime or the urine or feces of rats.

Uhlenhuth and Zeulzer [24] showed that there is some immunologic relationship between water leptospiras and those of Weil's disease, but that they are distinctly different species. On the other hand the water leptospiras acquire pathogenic properties by passing them through animal serum. Shiga [25] reported his extensive research on the relationships between Spirochaeta icterogenes and Spirochaeta pseudo-icterogenes. He concluded that the two strains are not related either immunologically or culturally.

Material and Methods.—Leptospiras were observed for the first time in preparations of the slime hanging at the end of a waste pipe in the laboratory ice box. In some preparations many leptospiras were present in each field, while but few were found in others. The slime was put into petri dishes containing 20 cc. of 2% human feces in tap water. Many active leptospiras were found in several petri dishes after 5 to 7 days' incubation at room temperature.

Water samples were cultured from the following sources: tap water, Boston; water from the melting ice in the refrigerator, rain puddles in the street; pools and ponds in Franklin Park, Boston; Jamaica Pond.

These water samples were collected in sterile containers, brought to the laboratory and distributed into sterile petri dishes. It was necessary to use at least 100 cc. of water for each sample. To each petri dish enough sterile (heated) fecal emulsion was added to give the medium a rich cloudy appearance. If too much or too little feces is added leptospiras may not develop. About 1 to 2% makes a good medium. Better growth was obtained in petri dishes than in tubes or in glass jars. The cultures were set at room temperature, in the dark, and examined weekly for five weeks. The sample was recorded positive if leptospiras were found in any dish.

Experimental.—These microorganisms, mixed with other bacteria, did not show growth after 18 days in 2% serum or 2% blood in 0.85% salt solution, but abundant growth was obtained in tap water

[23] Brit. M. J., 1924, 1, p. 313.
[24] Klin. Wchnschr., 1922, 1, p. 2124.
[25] Ztschr. f. Immuntätsforsch. u. Exper. Therap., 1924, 40, p. 148.

plus feces. Better growth was obtained in petri dishes than in test tubes on the same medium. With succeeding transplants the leptospiras, especially in pure cultures, seemed to grow equally well in test tubes as in petri dishes. While the leptospiras were still mixed with bacteria, rich mediums stimulated the multiplication of the latter, but the former failed to grow at all. After isolation of the leptospiras by filtration they grew luxuriantly on 4% rabbit serum. The leptospiras lived, but multiplied scantily at 31 to 35 C. The optimum temperature is 25 to 28 C. They survived for two hours at a temperature of 58 C.

Isolation into Pure Cultures.—Their isolation into pure cultures was accomplished by filtration through Berkefeld candle "N". The filter was prepared in the usual manner and numbered in order to keep record of its filtering qualities. Leptospiras free from other bacteria were obtained only in the following manner:

The glass mantle of the filter was filled with a 10 to 15% sterile emulsion of feces and allowed to filter by gravity for one hour. At this time the fecal emulsion was thrown away and the culture was poured in its place. The filter was raised up, and loopfuls of filtrate at the end of the nipple were transferred to culture medium every five minutes for at least two hours. The transplants were made into a sterile medium consisting of 1 to 2% feces in tap water. The cultures were examined under the dark field illumination after ten to twenty days of incubation at 27 C. Those tubes that contained leptospiras only were subcultured on meat infusion agar in order to determine if any contaminating bacteria were present. This was easily demonstrated because the leptospiras do not grow on agar; so that a blank transplant indicated the absence of bacteria. The pure cultures were carried on 1 or 2% fecal emulsion for two generations. With succeeding transplants, rabbit serum was added to the fecal emulsion. At first only 1% serum was used but gradually it was increased to 4%. Finally the use of the feces was discontinued and only rabbit serum in tap water was used with good results. Transplants were also carried in fecal emulsion plus serum as a precaution against losing the cultures.

The rate at which the leptospiras pass through the filter varies according to the porosity and the treatment of the candle. The shortest period in passing through the filter was fifteen minutes from the time the culture was put into the filter until the leptospiras were recovered in the filtrate. This, however, was the case only with filters that were not treated with fecal emulsion. The longest period for their passing through the filter was one hour and fifteen minutes. This was the case with filters that were first treated with fecal emulsion. On the other hand the spirochetes did not pass through tight filters even in four hours.

In all, 40 filtration experiments were performed and 37 pure cultures were obtained, from 4 different water samples. In one experiment, out of 33 transplants, five contained only leptospiras; while only

one pure culture was obtained from seven transplants in another filtration. In one experiment, out of 40 transplants, 12 were free from contamination. In another experiment, out of 48 transplants, 19 were free from bacteria.

According to my experience very few Berkefeld filters are suitable for separating spirochetes from bacteria. Only two filters out of twenty proved effective for this purpose. Each candle must be tested before its filtering qualities can be determined.

Survey of Municipal Drinking Water.—Since leptospiras seemed so widely distributed studies of the drinking water of several cities were undertaken. Water from 40 cities was obtained and treated in the following manner:

Two or three petri dishes were nearly filled with the sample, while the remainder of the water was left in the jar. Sterile fecal emulsion, enough to give a rich turbid appearance, was added to each dish and to the jar. Varying the amount of fecal emulsion in each dish was useful, because the correct amount varied somewhat. The cultures were set at room temperature and were protected from strong sunlight. Weekly examinations, under the dark field, were made for six weeks. Out of the 40 samples, 35 or 87.5% were positive; while only 5 or 12.5% were negative for leptospiras.

Altogether, from all sources, 64 water samples were cultured and examined: 44 (69%) were positive, and 20 (31%) were negative for leptospiras.

A correlation between the method of filtering and treating the water in the various cities shows that slow sand filtration plus the use of chlorine eliminates the leptospiras from the drinking water (Providence, Rhode Island; Lawrence, Massachusetts, and Hartford, Connecticut). On the other hand, New Haven, uses slow sand filtration and chlorine yet leptospiras were found in great numbers, in a sample examined. One is more likely to find them, however, in water which has not been sand filtered or treated with chlorine.

Animal Experiments.—Young guinea-pigs weighing 160 grams were used because they are more susceptible to infection. Inoculation was done by mouth, eye, ear, rectum, and subcutaneously, but the intraperitoneal method was employed most frequently. The doses varied from 0.5 cc. to 6 cc. of the cultures. In a series of experiments daily massive doses were given, but no symptoms of illness developed. Animals immunized with these leptospiras are not protected against a virulent strain of Leptospira icterohemorrhagiae.

Leptospira cultures were put into the drinking water of six white mice, but none of them became infected, as was shown by the negative findings in their urine. Intraperitoneal injections of heavy doses failed to infect the mice. Injections of guinea-pigs with ground liver and kidneys from these mice did not produce jaundice.

The immune serum produced by a virulent strain of Leptospira icterohemorrhagiae does not agglutinate the water leptospiras. They are agglutinated by their hemologous serum.

Morphology.—The leptospiras as found in their natural habitat have very fine and closely wound spirals. The cell body is from 5 to 7 microns long, 0.2 microns wide, with one or both ends hooked. When grown on artificial medium they are from 5 to 45 microns long, 0.25 microns wide. The spiral amplitude is longer and the coils appear loosely arranged. Their fine spirals and curved ends definitely place them in the leptospira group.

Multiplication is by transverse division and the process is readily observed. The fully grown leptospira begins to bend in hingelike fashion at the point of division. The middle bends to and from, while the ends twist about each other, then unwind, straighten out and twist again (fig. 8). This process is similar to what Gross [26] has described as "incurvation" in Cristospira pectinis. Either one or both ends are hooked (figs. 1, 2, 3, 4). When they are actively motile they appear like figures 5, 6, 7 or 8. They rotate on the long axis and have no anterior-posterior polarity.

They do not stain readily. In stained preparations they lose their morphological characteristics (figs. 17-21).

The most satisfactory staining method is as follows: The culture is spread very thinly on a clean glass slide and immediately exposed for one-half minute to the fumes of 1% osmic acid, air-dried, then immersed for 30 minutes in Fontana's solution 1 (acetic acid, 1 part; formalin, 2 parts; distilled water, 100 parts). The preparation is washed thoroughly in water then placed for 4½ hours in a mixture of 1 cc. stock solution of basic carbolfuchsin and 1 cc. saturated alcoholic solution of gentian violet in 50 cc. distilled water. The preparation is again washed thoroughly in water from 5 to 30 minutes, rinsed, air-dried and examined. Exposure to osmic acid is not necessary although it is helpful in preserving the morphological characteristics of the organisms.

SUMMARY

Leptospira biflexa is widely distributed in potable waters in Eastern United States. It was found in 69% of all water samples examined and

[26] Mitt. Zool. Sta., Naepel., 1910. 20. p. 1.

PLATE 1
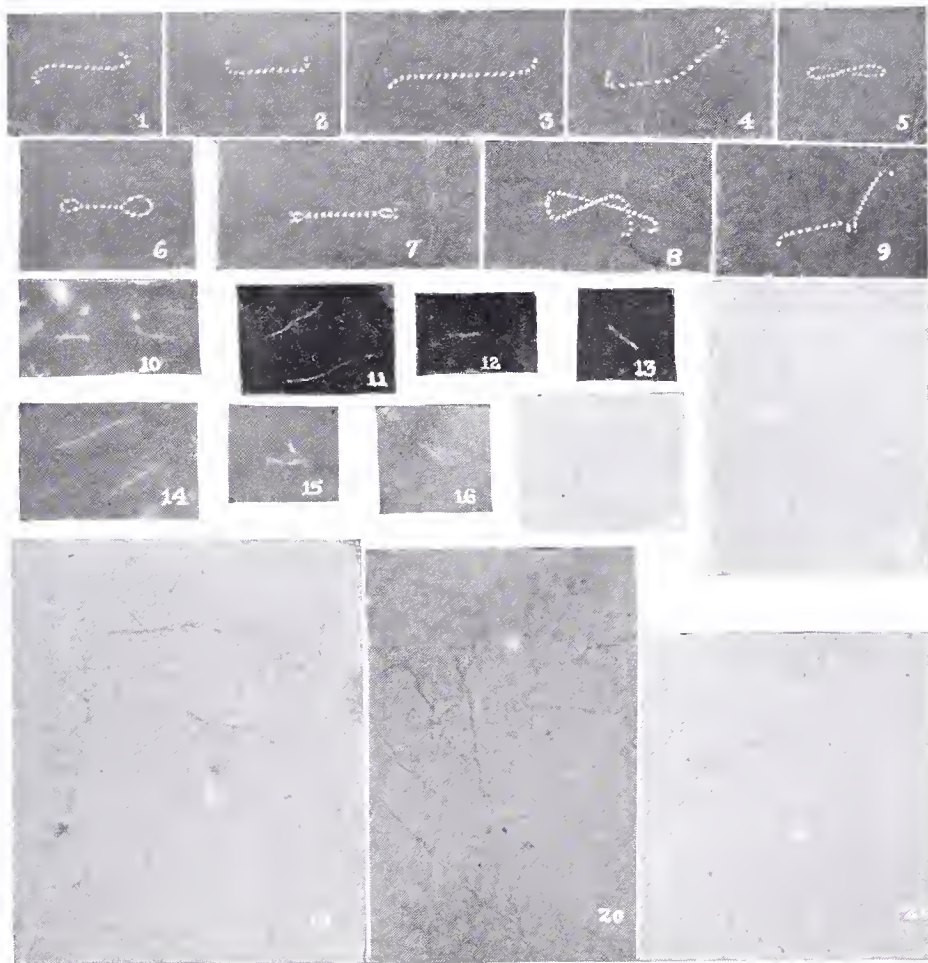
Figs. 1-9.—Drawings of Leptospira communis as observed under dark field illumination. Figures 1-4 represent different positions of spirochete; figures 5, 6 and 8 show how a single organism doubles up and twists its end during fission; figure 7 represents a rapidly moving leptospira; and figure 9 shows the process of transverse division.

Figs. 10-16.—Dark field illumination microphotographs of varying magnifications.

Figs. 17-21.—Stained preparations from 8 day cultures grown on 4% rabbit serum in tap water.

in 87.5% of municipal drinking water from 47 cities. There is no reason to believe that the European water leptospiras represent a different species. This wide distribution and the value of feces in initial culture suggests that like the colon bacillus one of the natural habitats of this genus is in the intestinal tract of animals. If the rules of nomenclature permitted, Leptospira communis would be an appropriate name.

This leptospira is not pathogenic for mice and guinea-pigs. It does not protect against infection with Leptospira icterohemorrhagiae. The value of filtration through candles made of diatomaceous earth is established as a useful procedure as an aid to their isolation in pure culture.

SOLUBLE ANTIGENS OF BACTERIUM ENTERITIDIS *

Sara E. Branham and Eleanor M. Humphreys

From the Department of Hygiene and Bacteriology, the University of Chicago

A previous paper by one of us on the toxic properties of products of Bacterium enteritidis [1] described experiments in which the "toxins" used were the filtrates of cultures grown in a simple synthetic medium. The toxic principle of these filtrates seemed to be identical with that obtained when the microorganisms were grown in veal infusion and peptone broths, both in the symptom complex and the pathology produced by injections in animals. Serum from rabbits immunized with these synthetic medium filtrates protected other rabbits against the toxic effects of both the synthetic and the peptone broth filtrates. Conversely, serum from rabbits immunized with broth filtrates also protected against both preparations.

Several investigators, using meat-peptone-broth filtrates, have reported producing antitoxic serums with various members of the paratyphoid group. Ecker [2] and Franchetti [3] demonstrated agglutinins in the antitoxin they produced. Franchetti reported complement-fixing antibodies. Our synthetic medium filtrates seemed favorable material with which to investigate the production of various antibodies since they contained nothing except known salts and bacterial products.

Technic.—The strain of Bacterium enteritidis used was an old stock culture (E52 of Prof. Jordan's collection) of relatively low virulence; but it produced very toxic filtrates. The virulence of this strain was increased by passage through rabbits. Care was taken to identify the recovered organism as Bact. enteritidis after each passage. After the 11th passage 1/10 of an agar slant culture killed a fully grown rabbit in less than 24 hours. This strain was used for all subsequent work.

The medium [4] used consisted of: ammonium lactate 0.6%; K_2HPO_4 0.2%; and NaCl 0.5% at a P_H of 6.8 to 7.2. In our later experiments ammonium succinate was substituted for the lactate since solutions containing ammonium lactate cannot be evaporated to dryness. Inoculations were made into 500 cc. quantities of the medium contained in 1000 cc. flasks.

Bact. enteritidis was grown in the above medium at 37 C. for 6 to 14 days. The cultures were then passed through Berkefeld N filters, and the filtrates tested

[1] Branham: J. Infect. Dis., 1925, 37, p. 291.
[2] J. Infect. Dis., 1917, 21, p. 541.
[3] Ztschr. f. Hyg. u. Infektionskrankh., 1908, 60, p. 127.
[4] Braum and Cahn-Bronner: Centralbl. f. Bakteriol., I. O., 1921, 86, p. 1.

for sterility and for toxicity. As far as possible all work was done with filtrates, 2 cc. of which, given intravenously, killed a rabbit weighing one kilogram within 2 to 10 hours.

In the agglutination tests serum dilutions and suspensions of the organisms were made in 0.5% salt solution, and all tests incubated at 37 C. in a water bath for 2 hours and then placed in an icebox overnight.

Precipitin tests were carried out in the usual manner. The tubes were incubated for 30 minutes in a water bath at 37 C. and read at once.

Several methods of preparing bacterial antigens for complement fixation were tried. The method of Melick[5] was finally adopted since it gives an antigen that is free from anticomplementary action. Heavy suspensions of washed organisms were placed in special silvered tubes and subjected to freezing and thawing 15 to 25 times in rapid succession by means of liquid air and boiling water. The clear supernatant fluid obtained after centrifugalization was used as an antigen. Anti-sheep amboceptor, a 5% suspension of sheep cells, and 0.85% salt solution were used. Best results were obtained with 1½ units of complement, 0.05 cc. of the serum to be tested, and 0.5 cc. of the undiluted antigen. These tests were carefully controlled.

TOXIC FILTRATES AS ANTIGENS

Rabbits were immunized with the toxic filtrates by intravenous injections every second day, beginning with a very small quantity and increasing it until the animals were receiving several times the fatal dose. Much care was necessary in this immunization because of the variation in individual susceptibility among rabbits. Three series of 12 rabbits each were immunized with the filtrates. Immunization of series 1 and 2 was carried on for about 6 weeks. The immunization of series 3 extended only over 17 to 18 days. Series 1 was used only in the protection experiments described elsewhere.[1]

Serums obtained from these immunized rabbits protected others against the toxic effects of the filtrates.[1] The protection afforded by these antiserums, though weak, was definite. Against some filtrates the protection was complete with the amounts used; with others it was relatively slight. Although there was always evidence of some degree of protection beyond that afforded by the normal serums used as controls, the results were variable. We were not convinced that a true antitoxin had been produced. The new results obtained in this antibody study (summarized in table 1) are as follows.

All immune serums agglutinated Bacterium enteritidis. Control tests carried out with other organisms or with normal serums were consistently negative. Tests were made for specific precipitating substances in the serums of the immunized rabbits, using as antigens filtrates of cultures grown in the synthetic medium. In most cases, though not invariably, a definite precipitate appeared as a ring at the line of contact between serum and antigen. This never occurred

[5] J. M. Res., 1922, 43, p. 405.

in tests made with the normal serum. With an antigen prepared from Bact. enteritidis by Meliek's method complement-fixation was demonstrated with 11 of the 13 immune serums tested. Results with the normal serums were consistently negative.

The antitoxic value of these serums varied greatly, and seemed to bear little relation to the concentration of other antibodies present. For example, serum 5, which had good agglutinating and precipitating titers, but did not fix complement, protected as well as serum 9, which, however, fixed complement and had an agglutinin titer more than twice as high. It protected better than serum 6 which had as high an agglutinin titer and a "2 plus" complement-fixation, though the precipitin titer was low. The serums of series 3 were too weak to afford protection in the amounts available for use.

TABLE 1

Demonstrable Antibodies in the Serum of Rabbits Immunized with Toxic Filtrates

Serums		Agglu-tinin Titer	Precip-itin Titer	Comple-ment Fixation	Antitoxin (Animal Protection Tests)
Number	Kind				
1, 2, 3	Normal....................	0	0	0	0
4	Immune (whole bacteria)....	3,000	0	0	—
5	Immune....................	3,000	80	0	Complete protection
6	Immune....................	3,000	20	++	No protection
7	Immune....................	2,000	80	nat.amb.	Partial protection
8	Immune....................	3,000	40	++	Complete protection
9	Immune....................	10,000	20	+	Complete protection
10	Immune....................	100	+	+	—
11	Immune....................	200	+	+	—
12	Immune....................	800	0	++	—
13	Immune....................	400	+	+++	—
14	Immune....................	10	+	+	—
15	Immune....................	400	+	+++	—
16	Immune....................	200	0	+	—
17	Immune....................	800	+	+	—
18	Immune (whole bacteria)....	400	0	0	—
19	Normal....................	0	0	0	—

Series 2: Rabbits immunized for 6 weeks — rows 4–9.
Series 3: Rabbits immunized for 17 to 18 days — rows 10–19.

Signs 0 and — indicate, respectively, negative results and no test made, in all tables.

THE QUESTION OF A NONPROTEIN ANTIGEN

These preliminary experiments showed that in the serums of animals immunized with sterile filtrates of cultures of Bact. enteritidis grown in a synthetic medium, there may have been antitoxins, as evidenced by the protection shown against the toxic filtrates; there were unquestionably agglutinins, precipitins, and complement-fixing antibodies. These animals had received only sterile bacteria-free filtrates, in which neither bacterial protein nor protein split products could be detected by any of the usual tests. Not only the biuret test, but the Molisch test for a carbohydrate radical, the Millon test for tyrosine, Ehrlich's and the vanillin tests for tryptophane, and the ninhydrin test were all negative. The diazo test for histidine could not be used here because of the presence of interfering substances.

Antibody production in animals treated with nonprotein antigen has been reported by a number of investigators. Most of this work has been done with material of a lipoidal nature, but antigens of other types have also been described. In some of the older work a nonprotein nature is claimed for a true antigen on the ground that it does not give a biuret reaction.[6] Evidence of antigenic value for materials apparently protein-free is reported by Nicolle,[7] Zunz,[8] Ferry and Fisher,[9] and Perlzweig and Steffen.[10] Ford[11] has reported producing a protective serum for the glucoside of Rhus toxicodendron and an antihemolytic serum in rabbits with a hemolytic glucoside from Amanita phalloides.

We were unable to detect any substance of protein nature in the antigen we used in our experiments described above. Yet it seemed reasonable to suppose that some dissolved bacterial protein might be present in a quantity sufficient to serve as an antigen. Consequently methods of concentration were adopted.

Method 1.—Ten liters of toxic filtrate were concentrated in vacuo at a temperature not exceeding 40 C. to a syrupy fluid with a volume of 200 cc. This concentrated material was examined for protein and protein split products by the biuret, Millon, Ehrlich's, vanillin, and ninhydrin tests. As with the original toxic filtrates, substances were present that interfered with the diazo reaction, but tests with the other reagents were negative.

Further separation of any protein in this concentrated filtrate was undertaken by adding 4 or 5 volumes of alcohol to one portion, drying the precipitate, and testing it with the same reagents used before. All tests were negative. Phosphotungstic acid was used as a precipitant with another portion and the precipitate tested with the same reagents. Again the results were negative. With this material it was possible to conduct the diazo test because the interfering substances had been removed during the phosphotungstic acid treatment. The test was negative.

Method 2.—By substituting ammonium succinate for ammonium lactate it was now possible to evaporate the toxic filtrates to dryness. Nineteen liters were thus evaporated in vacuo at 40 C., the residue dried in a vacuum desiccator, and the solid, slightly hygroscopic mass ground to a fine creamy white powder. It is necessary to be very careful of the P_H of the material during evaporation; ammonia goes over rapidly, the acid concentration becomes greater, and charring results. This material gave negative biuret, Millon, vanillin, Ehrlich's, and Molisch tests. A vividly positive ninhydrin test was found later to be due to the changed ammonium salts present.

This residue was still toxic for rabbits, but less so than the filtrate in proportion to its concentration. As much as 0.5 to 0.7 grams were required to kill a rabbit weighing 1 kilogram within 2 to 10 hours. This residue is referred to in subsequent experiments as "toxic residue."

THE TOXIC RESIDUE AS AN ANTIGEN

Determination of the antigenic properties of this toxic residue was now begun. Intravenous injection of rabbits with this material was undertaken with misgivings as it was feared that the salts would prove

[6] Beitr. z. Chem. Phys. u. Path., 1902, 1, p. 397.
[7] Ann. de l'Inst. Pasteur, 1898, 12, p. 161.
[8] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1913, 16, p. 580.
[9] Brit. J. Exper. Path., 1924, 5, p. 185; J. Lab. & Clin. Med., 1925, 10, p. 787.
[10] J. Exper. Med., 1923, 38, p. 163.
[11] J. Infect. Dis., 1906, 3, p. 191; 1907, 4, p. 541; J. Pharmacol., 1910, 2, p. 145.

too toxic. Removal of the salts by dialysis did not seem advisable at this time, therefore control material was prepared by evaporating 2 liters of the uninoculated medium to dryness, thus obtaining a crystalline white material. The largest amount of this nontoxic residue which could be well borne by fully grown rabbits was determined, and all doses given in immunization were kept below this amount. To make sure that there were no degenerative changes produced by the salts, and also to obtain serums for controls, several rabbits were treated with this nontoxic material instead of the toxic residue by the progressive method. The materials for injection were prepared by weighing out the desired amounts, dissolving these in freshly distilled water, and injecting them very slowly. The animals bore the injections well when their weight and general condition were carefully watched and the doses spaced

TABLE 2

Demonstrable Antibodies in the Serum of Rabbits Immunized with Concentrated Residues

Serum	Immunizing Material	Agglutinin Titer	Precipitin Titer	Complement Fixation	Antitoxin (Animal Protection Test)
A and B	(Normal)...........	0	0	0	0
C	Whole bacteria....	2,000	0	+	0
D	Whole bacteria...	200	0	Anticomplementary	0
E	Toxic residue......	2,000	20	+	—
F	Toxic residue......	3,000	0	0	—
G	Toxic resdiue......	5,000	10	+ +	—
H	Toxic residue......	3,000	40	+ +	—
I	Toxic residue......	4,000	40	+ + + +	—
J	Toxic residue......	4,000	100	+ + + +	Complete protection
K	Toxic residue......	4,000	100	+ + + +	Complete protection
L	Toxic residue......	4,000	100	+ +	Partial protection
M	Toxic residue......	4,000	100	+ + + +	Partial protection
N	Nontoxic residue...	0	0	0	0

accordingly. The amounts given began with 0.1 gm. (equivalent to about 7.5 cc. of the filtrate) and were increased gradually to 1.0 gm. (equivalent to about 75 cc. of the filtrate).

After 8 weeks the animals were bled and their serums tested for agglutinins, precipitins, and complement-fixing antibodies. The serums of those rabbits given the nontoxic residue, of rabbits immunized with Bact. enteritidis organisms, and of normal rabbits were used as controls. The results of these tests for demonstrable antibodies in the serums of this series of rabbits are summarized in table 2.

Agglutinins for Bact. enteritidis were present in the serums of all rabbits immunized with the toxic residue; to a less degree in those of rabbits immunized for the same length of time with Bact. enteritidis. In those of normal rabbits and of rabbits given the nontoxic residue there were no agglutinins demonstrable.

The salt residues, both toxic and nontoxic, were obviously unsuitable to use as antigens for the precipitin tests on account of the hypertonicity of the resulting

solutions. Two antigens were found highly satisfactory: the unconcentrated toxic filtrate; and the solution obtained by alternately freezing and thawing heavy suspensions of Bact. enteritidis, prepared for use in the complement-fixation tests.[5] With these antigens precipitins were demonstrated in the serums of all rabbits immunized with the toxic residue. Precipitins were not demonstrable in the normal serums tested, in serums from animals treated with the nontoxic residue, nor in those from rabbits immunized with Bact. enteritidis.

Complement was fixed by the serums of all toxic residue immunized rabbits, and this fixation was complete in 4 of the 9 serums of the toxic residue series. Serums of normal animals and of those given the nontoxic residue failed to show complement fixation. Serum from one of the rabbits immunized with the whole Bact. enteritidis gave a slight fixation; the other was anticomplementary.

Serums J and K protected perfectly against a toxic filtrate, 2 cc. of which killed normal rabbits within 24 hours. L and M could also protect rabbits, but not as completely as J and K. Control serums A, B, C, D, and N gave no protection; E, F, G, H, and I were not tested for antitoxic action.

The most striking thing about the above results is the excess of antibody production in the serums of those rabbits immunized with the toxic residue over that of those from rabbits receiving the intact organisms. The value of soluble products of certain bacteria as antigens has been indicated by Ferry and Fisher,[9] by Perlzweig and Steffen,[10] and by Seibert.[12] In our own work with Bact. enteritidis we have found them to be distinctly superior to the whole organism, especially when concentrated.

EXPERIMENTS WITH DIALYZED RESIDUE

Attempts were made to demonstrate anaphylaxis in guinea-pigs by the Schultz-Dale method. The salts of the toxic residue had such a pronounced effect upon the uterine muscle that it was evident that they would have to be eliminated by dialysis before these experiments could be carried on. The method of continuous dialysis with a constant volume of water devised by Hanke and Koessler [13] was employed. The dialysis was repeated with fresh supplies of distilled water until direct Nesslerization failed to reveal nitrogen in the dialyzing sac and tests for Cl and PO_4 were negative. A heavy grayish precipitate settled in the bottom of the sac, leaving a faintly opalescent fluid above it.

Neither the sediment nor the supernatant fluid gave any of the usual tests for protein or protein split products; biuret, Millon, ninhydrin, vanillin, Ehrlich's or diazo reactions. The highly insoluble gray precipitate gave a brilliant Molisch reaction, which suggests that it contains or is a carbohydrate. Further investigation of this substance obtained from Bact. enteritidis is now in progress.

[12] Am. J. Physiol., 1925, 71, p. 621.
[13] Hanke and Koessler: J. Biol. Chem., 1925, 66, p. 495.

The opalescent supernatant fluid, which gave a specific precipitin reaction with Bact. enteritidis antiserums, was now used as an antigen in attempting to produce anaphylaxis in guinea-pigs. Virgin guinea-pigs, 2 to 4 weeks old, were given sensitizing doses of 0.1, 0.2, or 0.4 gm. of the toxic residue intraperitoneally. After about 2 weeks the uterine strip method was used to determine their sensitization. Definite contractions, apparently identical with those occurring in true anaphylaxis, occurred when the dialyzed material came in contact with the strip of uterine muscle from the guinea-pigs sensitized with the larger amounts. These contractions did not occur with the uteri of normal guinea-pigs, or of guinea-pigs which had been given the nontoxic residue. In one respect these reactions failed to meet the criteria established for true anaphylactic shock: the muscle was not desensitized, even by several contacts with the antigen. Whether this was due to the weakness of the antigen, making the desensitization a gradual thing, or whether it was not true anaphylaxis is uncertain, and we do not feel warranted in claiming that we have demonstrated anaphylaxis with this material.

Amounts of this opalescent fluid equivalent to as much as 500 to 700 cc. of the unconcentrated toxic filtrates produced no symptoms when given intravenously to rabbits.

The remainder of the opalescent fluid obtained by dialysis was now concentrated in vacuo at 30 to 40 C to a volume of about 5 cc. This was again tested for protein by the biuret, Millon, ninhydrin, vanillin, and diazo tests. The ninhydrin, vanillin, and diazo tests were very faintly, but definitely, positive. The Millon test was negative, possibly because it is less sensitive.

The vanillin, diazo, and ninhydrin reactions are, of course, tests for certain constituents in the protein molecule rather than for whole protein. Proteoses, peptones, and amino acids are, however, readily dialyzable; therefore these positive tests indicate that protein may have been present.

The diazo test for histidine has been found [11] to be sensitive enough to detect histidine in a dilution of 1 : 1,000,000. The vanillin test will give a definitely positive reaction with a 1 : 1,000,000 dilution of tryptophane. If we assume a 2% concentration of histidine or tryptophane in the protein molecule these tests may be interpreted to indicate a maximum protein concentration of 1 : 20,000. The 1 cc. of concentrated dialysate used in each test represented about 500 cc. of filtrate. Therefore, in the original solution the protein dilution was at least

[11] Koessler and Hanke: J. Biol. Chem., 1919, 39, p. 497.

1 : 10,000,000, or 0.000,000,1 gm. per cc. of filtrate. Rabbits immunized with filtrates received 30 to 40 cc., or about 0.000,003 to 0.000,004 gm. of protein in all. Rabbits immunized with the concentrated material received 8 to 13 gm. of the toxic residue. One gram of this solid material was equivalent to 75 cc. of the filtrate, or about 0,000,007,5 gram of protein. Thus the animals were given amounts corresponding to 600 to 1,000 cc. of the original material, or 0.000,06 to 0.000,10 gram of protein.

Very little work has seemingly been done on the determination of the amounts of protein necessary to stimulate antibody formation. Wells[15] succeeded in producing fatal sensitization of guinea-pigs with single doses of crystallized egg albumin as small as 0.000,001 of a gram. Osborne, Mendel, and Harris[16] reported that although 0.004 gram of their purified ricin proved fatal to medium sized rabbits, less amounts conferred an immunity to this dose. The results of Seibert[12] are more comparable to those reported in this paper since they were obtained with bacterial products. By immunizing rabbits with concentrated pyrogenic distilled water she demonstrated agglutinins for a microorganism which she isolated from the distilled water. She determined the amount of nitrogen in another sample by a micro-nitrogen method, and on the basis of the figures obtained calculated that this water, unconcentrated, would have a protein content of 0.000,000,05 gm. per cc. Thus her immunized animals received a total of 0.000,062 to 0.000,118 gm. protein.

From calculations based on the fact that our final material tested gave positive amino acid tests comparable with those obtained with 1 : 1,000,000 solutions of trytophane and histidine, our animals immunized with filtrates received 0.000,003 to 0.000,004 gm. of protein. These figures are, of course, an approximation.

We did not have enough material left for a quantitative micro-nitrogen determination. Since our medium contained an ammonium salt such a procedure would have been of doubtful value, at most. Although we dialyzed our material until we could no longer detect ammonia by Nesslerization, the subsequent concentration of this dialysate would have also resulted in the concentration of traces of ammonia that might possibly have remained in the sac. The accuracy of our results in a micro-nitrogen determination would have been open to question.

SUMMARY

Bacterium enteritidis can be cultivated in a synthetic medium free from protein. Berkefeld filtrates, free from bacteria, obtained after 6 to 14 days incubation, are extremely toxic for rabbits when given

[15] The Chemical Aspects of Immunity, 1925, p. 196.
[16] Am. J. Physiol., 1905, 14, p. 259.

intravenously. These filtrates, when injected into rabbits, stimulate the production of agglutinins, precipitins, complement-fixing antibodies, and possibly antitoxins.

The antigenic properties of these filtrates are increased when the liquids are concentrated by evaporation in vacuo. The products so obtained are better antigens than suspensions of Bact. enteritidis.

Protein cannot be demonstrated even in the dry residue obtained by the evaporation of such a filtrate.

An opalescent fluid and a gray precipitate are obtained when this dry residue is dialyzed. Protein cannot be demonstrated in either of these fractions. The precipitate contains carbohydrate.

Further concentration of the opalescent fluid gives a liquid that gives very faintly positive reactions for tryptophane and histidine, and a faintly positive ninhydrin reaction. Protein was, therefore, possibly present.

An amount of filtrate that could, at most, have contained 0.000,003 to 0.000,004 gm. of protein led to definite antibody formation. Whether or not so small an amount of protein can, in fact, be held exclusively responsible for this antibody production may be questioned. Such a possibility should, however, be borne in mind in interpreting results obtained with apparently protein-free materials.

GROWTH OF CL. BOTULINUM AND CL. SPOROGENES IN VEAL INFUSION BROTH UNDER REDUCED PRESSURE *

Gail M. Dack, W. A. Starin and Marie Werner

From the Department of Hygiene and Bacteriology of the University of Chicago

Delayed germination of Cl. botulinum in various anaerobic mediums has been commonly observed by laboratory workers. Spores of Cl. botulinum not infrequently remain dormant over long periods of time. No less common is the occurrence of botulinum toxin in one can of food and not in others of the same lot subjected to exactly the same treatment. The presence of other bacteria,[1] the application of heat,[2] the effect of the amount of inoculum,[3] and a change in the hydrogen-ion concentration of the medium used,[4] have been shown to be factors influencing the growth and toxin production of Cl. botulinum. A possible factor which has received little attention is the effect of various gases and reduced pressures on the germination of spores and toxin production of Cl. botulinum.

A study was made first of the growth of Cl. botulinum types A and B and of Cl. sporogenes under reduced atmospheric pressures. After this preliminary determination, the gases oxygen and carbon dioxide were placed in the evacuated tubes under various reduced pressures.

Spores and vegetative cells were used in inoculating the medium. All the strains used were from single-cell cultures and were tested for purity. The spores were obtained by growth of the organisms in veal infusion broth containing 2 per cent peptone and 0.5 per cent sodium chloride with a P_H of 7.0 to 7.2. They were washed with sterile 0.85 per cent salt solution by means of centrifugalization. The suspensions were heated for ten minutes at 80 C. to destroy any toxin that might remain. Such heating would serve to kill the less resistant spores and the vegetative cells.

Vegetative cells were obtained by growth in the same kind of broth as was used in growing the spores. Twenty-four hour cultures were used so that daily transfers were necessary. The cells were washed and suspended in sterile 0.85 per cent salt solution but unlike the spore suspensions were not heated.

Suspensions of spores and vegetative cells were counted by the hemocytometer method. At first both a plate count and a hemocytometer count were made but the results were so at variance that the latter method only was chosen and used throughout the entire experiment.

1 Jordan, E. O., and Dack, G. M.: J. Infect. Dis., 1924, 35, p. 576.
2 Dickson, E. C., and Burke, G.: Ibid., 1925, 36, p. 472.
3 Starin, W. A.: J. Infect. Dis., 1926, 38, p. 101.
4 Dozier, C. C.: J. Infect. Dis., 1924, 35, p. 105.

One-tenth cc. quantities of the counted suspensions were inoculated into 2 cc. of veal infusion broth of the same lot that was used in growing the spores and vegetative cells. The broth was filtered several times to clear it so that growth could be determined by turbidity. The suspensions were standardized in later experiments to contain 50 million organisms per cc. By means of this method results of one series of experiments were more comparable with those of another.

The tubes containing the broth plus the inoculum were constricted in the center by means of a blast flame. Tubes drawn out in this manner were sealed readily at the constriction with a small hand flame. The tubes containing broth plus a known number of organisms were then subjected to various reduced pressures.

The apparatus designed for this part of the experiment consisted of twelve T tubes joined with pressure tubing. The termination of each T tube connec-

TABLE 1

Growth of Spores of Cl. Botulinum A and B and Cl. Sporogenes at Reduced Pressures

Spores	Tube	Days for Growth, at Different Pressures (Cm. Mercury)											
		0.8	0.85	0.9	4.0	5.0	8.0	10	11	12	13	15	16*
A........................	1	1	2	2	1	0	14	0	3	60	60	19	49
	2	1	2	2	1	13	25	0	5	60	60	0	75
	3	1	2	—	—	0	—	0	—	—	—	—	—
	4	1	2	—	—	0	—	0	—	—	—	—	—
B........................	1	1	1	2	1	0	29	0	—	—	—	0	0
	2	1	1	2	1	5	0	0	—	—	—	0	0
	3	1	1	—	—	0	—	0	—	—	—	—	—
	4	1	1	—	—	1	—	0	—	—	—	—	—
C........................	1	1	1	2	1	2	0	0	—	—	—	0	0
	2	1	1	2	2	2	0	0	—	—	—	0	0
	3	1	1	—	—	2	—	0	—	—	—	—	—
	4	1	1	—	—	2	—	0	—	—	—	—	—

* Growth did not occur at pressures 20, 21 and 26 cm. Signs 0 and — indicate, respectively, no growth, and no test made.

In this and the succeeding tables, A refers to Cl. botulinum A, B to Cl. botulinum B, and C to Cl. sporogenes.

tion was fitted with a one-hole rubber stopper. The series of tubes connected in this manner was evacuated with an oil pump. The desired pressures were obtained by means of two manometers inserted in the series. One was used in registering high pressures, the other low pressures. All connections were made air tight with sealing wax and Dekotinsky's cement. The entire apparatus was covered with shellac making the whole as airproof as possible.

A preliminary experiment was made using pressures varying from 0.8 cm. to 26 cm. of mercury. Eight-tenths cm. was below the vapor tension of the broth but represented the manometer reading obtained when the pump was operating and before an equilibrium was reached. The results are shown in table 1. Without exception growth was uniformly obtained at pressures of 4 cm. or less. At the highest pressures (8-20 cm.) there appears to be no uniformity of growth. In one instance growth did not occur until after 75 days of incubation.

Oxygen was substituted for air at pressures of 5 and 10 cm. The tubes containing broth plus spores were first evacuated for one-half hour. Oxygen was then allowed to pass in to the desired pressure, at which pressure the tubes were allowed to remain for one hour. After a period of almost three months no growth was observed. The failure to produce growth in an oxygen environment may be due to the production of hydrogen peroxide by the bacteria. McLeod and Gordon believed that the inhibitory substance developed in pneumococcal cultures, to which there is abundant access of oxygen, is hydrogen peroxide.[5] The same writers also showed that anaerobes cannot tolerate more than very slight concentrations of oxygen because they produce hydrogen peroxide as soon as oxygen is available, and since they are very sensitive to this substance they die.[6]

TABLE 2

GROWTH OF CL. BOTULINUM A AND B AND CL. SPOROGENES AT REDUCED PRESSURES

Organism	Tube	Days for Growth at Different Pressures			
		Vegetative		Spores	
		0.8 cm.	5.0 cm.	0.8 cm.	5.0 cm.
A	1	3	6	2	5
	2	2	4	2	5
	3	2	0	2	5
	4	2	—	2	5
B	1	1	1	2	5
	2	1	1	2	5
	3	1	1	2	5
	4	1	1	2	5
C	1	1	2	4	2
	2	1	2	1	2
	3	1	2	1	1
	4	1	2	2	1

As a preliminary test, three cultures under varying reduced pressures, were tested for the presence of hydrogen peroxide by the benzidene test. None of the three cultures tested gave any color reaction. In a second series of experiments, tubes of broth containing vegetative cells were subjected to 0.8, 5, 10, 15 and 20 cm. of pressure and held at those pressures for 30 minutes. At the end of this time, the tubes were sealed at the constriction, incubated at 37 C. and observed over a period of about two months for growth. Broth containing spores was subjected to the same treatment. The second series (table 2) showed that spores and vegetative cells grow equally well under both 0.8 cm. and 5.0 cm. of air

[5] Biochem. J., 1922, 16, p. 499.
[6] J. Path. & Bact., 1923, 26, p. 332.

pressure. No growth resulted under pressures of 10, 15 and 20 cm Five to 7 days after growth, the tubes were opened and tested for the presence of aerobes, the production of toxin, increase in the number of organisms, the production of hydrogen peroxide and a change in the hydrogen-ion concentration of the medium. Throughout the entire experiment the hydrogen-ion concentration of the broth remained within the limits of growth for each organism tested. Hydrogen peroxide was not demonstrated in any of the tubes tested. The toxin was titrated by intraperitoneal injections of mice. One-half cc. of the 1 : 100 dilution proved fatal. Only in one instance was the titer as high as 1 : 1,000. No correlation could be made between the final count and the titer of the toxin produced.

TABLE 3

GROWTH OF CL. BOTULINUM A AND B AND CL. SPOROGENES AT REDUCED CARBON DIOXIDE PRESSURES

Organism	Tube	Days for Growth at Different CO_2 Pressures					
		Vegetative				Spores	
		5.0 cm.	15 cm.	25 cm.	35 cm.	35 cm.	50 cm.
A	1	1	2	2	3		2
	2	1	2	2	3	1	2
	3	2	2	2		4	2
	4	2	2	2	3	1	2
B	1	2	2	1	2	1	1
	2	2	2	1	2	1	1
	3	2	2	1	2	1	1
	4	2	1	1	2	1	1
C	1	2	2	1	6	1	1
	2	2	2	2		1	1
	3	2	2	2	4	1	1
	4	2	2	2	2	1	1

Much has been written regarding the influence of carbon dioxide on the growth of bacteria. The literature on this subject has been fully cited in an article by Rockwell [7] and since that time a number of other articles have appeared. Most of the publications, however, deal with the aerobes, few with the anaerobes. Inoculated broth tubes were subjected to various reduced pressures of carbon dioxide. The gas was passed through a solution of sodium pyrogallate to remove any oxygen that might be present. The tubes containing the broth plus the organisms were evacuated for 30 minutes and then the oxygen-free gas was allowed to enter to pressures of 5, 15, 25 and 35 cm. Growth resulted in all of the tubes. Pressures higher than 35 cm. were not used (table 3).

[7] J. Infect. Dis., 1923, 32, p. 98.

Spores were subjected to 35 and 50 cm. and growth was obtained at each of these pressures. Further work is necessary before the limits of growth under carbon dioxide pressures can be established.

Another series of tubes were evacuated for 30 minutes and carbon dioxide allowed to enter to atmospheric pressure. As the carbon dioxide was absorbed by the broth more of it was allowed to enter. At the end of one hour the broth was apparently saturated with the gas. The tubes were then evacuated to 5, 15, 25 and 35 cm. pressure and held for one hour, sealed and incubated. After a 5 to 7 day period of growth the broth was subjected to the same tests as were the other series in the experiment. Growth again resulted under all of the pressures. This

TABLE 4

Growth of Cl. Botulinum A and B and Cl. Sporogenes at Reduced Pressures After First Being Subjected to Atmospheric Pressure of CO_2

Organism, Vegetative	Tube	Days for Growth at Different CO_2 Pressures			
		5 cm.	15 cm.	25 cm.	35 cm.
A	1	1	1	2	2
	2	1	2	2	3
	3	1	2	2	3
	4	1	2	2	3
B	1	1	1	1	2
	2	1	1	2	2
	3	1	1	2	2
	4	1	1	2	2
C	1	1	1	2	2
	2	1	2	1	2
	3	1	1	2	2
	4	1	1	1	3

series compared very closely with the other carbon dioxide experiments (table 4).

Several tubes were tested not only after the 5 to 7 day interval of growth (table 5) but after 1, 5 and 30 days (table 6). The titer of toxin produced after one day was low, after five days the titer had increased considerably, and after thirty days the titer had fallen, in some cases below that of the first day. On the other hand, the final count after the one-day period of growth was higher than that after five days. After thirty days the count was the lowest of all. In cultures subjected to carbon dioxide pressures, the reduction in number of organisms after 30 days' growth was considerably greater than in cultures grown under air pressures.

TABLE 5

Growth and Toxin Production, Five to Seven Days After First Appearance of
Cloudiness in Broth

Organism	Tube	Pressure (Cm. Mercury)	Organisms, Millions per Cc.		Titer of Toxin in 0.5 Cc.	
			Inoculum	Final Count	Killed	Lived
Cultures (table 2) grown at reduced pressures (Vegetative)						
A.....	1	0.8	54.2	114.0	1:100	1:1000
	2	0.8	54.2	129.0	1:100	1:1000
	2	5.0	54.2	139.0	1:100	1:1000
B......	1	0.8	33.4	225.6	1:10	1:100
	2	0.8	33.4	240.0	1:10	1:100
	1	5.0	33.4	195.4	1:10	1:100
	2	5.0	33.4	190.4	1:10	1:100
C.......	1	0.8	39.2	115.4	—	—
	2	0.8	39.2	119.5	—	—
	1	5.0	39.2	146.0	—	—
	2	5.0	39.2	167.2	—	—
(Spores)						
A.......	1	0.8	50.0	181.8	1:100	1:1000
	1	5.0	50.0	166.4	1:1000	
B.....................	1	0.8	50.0	98.0	—	—
	1	5.0	50.0	253.6	1:50	1:100
C.....................	1	0.8	50.0	156.4	—	—
	1	5.0	50.0	157.0	—	—
Cultures (table 3) grown at reduced CO₂ pressures (Vegetative)						
A.....................	1	5.0	20.2	94.0	1:100	1:500
	1	15.0	20.2	150.8	1:500	1:1000
	1	25.0	20.2	422.8		
	1	35.0	20.2	112.6	1:500	1:1000
B.....................	1	5.0	8.7	156.2	1:50	1:100
	1	15.0	8.7	173.6	1:50	1:100
	1	25.0	8.7	237.0	1:50	1:100
	1	35.0	8.7	302.8	1:50	1:100
C.....................	1	5.0	12.5	162.0	—	—
	1	15.0	12.5	117.4	—	—
	1	25.0	12.5	148.8	—	—
	1	35.0	12.5	135.6	—	—
	3	35.0	12.5	170.0	—	—
(Spores)						
A.....................	2	35.0	50.0	224.0	1:10,000	—
	1	50.0	50.0	462.0	1:5,000	1:10,000
B.....................	1	35.0	50.0	236.0	1:500	1:1000
	1	50.0	50.0	269.4	1:10	1:100
C.....................	1	35.0	50.0	126.4	—	—
	1	50.0	50.0	366.8	—	—
Cultures (table 4) grown at reduced pressures after exposure to atmospheric pressure of CO₂ (Vegetative)						
A.....................	1	5.0	21.0	131.6	1:100	1:500
	1	15.0	21.0	122.6	1:500	1:1000
	1	25.0	21.0	115.2	1:500	1:1000
	1	35.0	21.0	120.2	1:500	1:1000
B.................	1	5.0	11.5	160.4	1:50	1:100
	1	15.0	11.5	211.2	1:50	1:100
	1	25.0	11.5	287.6	1:50	1:100
	1	35.0	11.5	207.6	1:50	1:100
C......	1	5.0	10.4	159.6	—	—
	1	15.0	10.4	83.8	—	—
	1	25.0	10.4	103.2	—	—
	1	35.0	10.4	100.2	—	—

Since tests had to be made on different tubes of broth, the second and third tubes might not contain the same number of organisms after the one day period of growth as the count of the first tube indicated. Another explanation which seems more logical since the same results were obtained in all of the tubes tested is that some of the organisms were lysed. A decrease in the titer of toxin after 30 days may be due to the formation of toxoids.

SUMMARY

Cl. botulinum types A and B and Cl. sporogenes were inoculated into tubes of veal infusion broth which were placed under reduced pressures of air, oxygen and carbon dioxide. The tubes were sealed under the

TABLE 6

Growth and Toxin Production One, Five and Thirty Days After First Appearance of Cloudiness in Broth

Organism	Tube	Pressure, Cm.	Period of Growth, Days	Organisms, Millions per Cc.		P_H	Titer of Toxin in 0.5 Ce.	
				Inoculum	Final Count		Killed	Lived
A	1	0.8	1	54.2	181.8	7.4	1:100	1:1000
	2	0.8	5	54.2	88.6	7.3	1:1000	1:5000
	3	0.8	30	54.2	65.2	7.0	1:100	1:500
Cultures with CO_2								
A	1	50.0	1	50.0	462.0	7.2	1:5000	—
	2	50.0	5	50.0	—	7.3	1:10,000	1:50,000
	3	50.0	30	50.0	4.3	7.4	1:1000	1:5000
B	1	50.0	1	50.0	209.4	7.2	1:10	1:50
	2	50.0	5	50.0	—	7.4	1:500	—
	3	50.0	30	50.0	1.3	7.0	1:1000	1:5000
C	1	50.0	1	50.0	366.8	7.0	—	—
	2	50.0	5	50.0	—	7.3	—	—
	3	50.0	30	50.0	5.0	7.2	—	—

reduced pressures and incubated at 37 C. The growth of the organisms and the toxin production of Cl. botulinum were studied under these conditions. Both spores and vegetative cells were used.

At air pressures of 4 cm. and less, growth was regular and occurred in most cases within one to two days. At pressures greater than 4 cm. growth was irregular and occurred in some cases after several months. No growth was obtained in any tube at pressures greater than 16 cm.

No marked difference was noted between the growth of spores and vegetative cells under reduced air pressures. Toxin was not demonstrated in greater dilutions where 0.8 cm. of pressure was employed than where 5 cm. was used.

Hydrogen peroxide was not demonstrated by Avery's method[8] in any of the tubes tested.

At oxygen pressures of 5 and 10 cm. no growth was noted after 3 months' observation.

Growth of the organisms occurred regularly and uniformly at carbon dioxide pressures as high as 50 cm. Pressures higher than this were not employed. Growth was no greater in tubes which were first evacuated and then placed under atmospheric pressure of carbon dioxide before finally reducing the pressures than in tubes which were merely evacuated and the final carbon dioxide pressure maintained.

Cultures tested after 1, 5 and 30 day periods of growth showed toxin production to be at its maximum after 5 days. The highest count of organisms was obtained after the 1 day period of growth.

The hydrogen-ion concentration of the cultures remained within the limits of growth for each organism tested throughout the entire experiment.

CONCLUSIONS

The degree of anaerobiosis is a factor influencing the delayed germination of the spores of Cl. botulinum types A and B. Oxygen pressures of 5 cm. completely inhibit the growth of Cl. botulinum, types A and B, and Cl. sporogenes. The organisms grow at 50 cm. pressure of carbon dioxide, and at this pressure toxin of a high titer is produced by Cl. botulinum types A and B.

[8] J. Exper. Med., 1924, 39, p. 275.

SPONTANEOUS INFECTIONS OF GUINEA-PIGS

PNEUMOCOCCUS, FRIEDLÄNDER BACILLUS AND PSEUDOTUBER-CULOSIS (EBERTHELLA CAVIAE)

ARNOLD BRANCH

From the Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau, N. Y., and The Hospital of the Rockefeller Institute for Medical Research, New York

During the course of the study of tuberculosis in small laboratory animals, one is repeatedly struck by the high percentage of common non-tuberculous infections which occur in such animals. In the usual prolonged experiments on tuberculosis in guinea-pigs, for example, death from intercurrent disease is very frequent. As these animals are chiefly used in the study of this disease, deaths from such intercurrent diseases may prevent one drawing any definite conclusions, or, indeed, the experiment may be completely spoiled. For this reason Dr. Petroff suggested that it would be advantageous to study such infections since no detailed studies have been made of these common infections in guinea-pigs comparable with those of Webster [1] on the respiratory infections of rabbits (B. lepisepticum), although the reports by Theobald Smith [2] and Holman [3] are extensive.

The observations here reported were made on a colony of about 400 guinea-pigs at Trudeau Sanatorium from February to June, 1926. Half of these ("old stock") had been bred on the premises, while the remainder ("new stock") were obtained from dealers in February. The epidemic was noticed in the first week of February when a larger number than customary of the animals began to die and many were observed to have snuffles and otitis. During the epizootic 114 animals died: 56 of these were infected with the Friedländer bacillus, 36 with the pneumococcus, 5 with B. bronchisepticus, 3 with streptococcus, 3 with organisms of the Eberthella group, and in the remainder the cause of death was undetermined.

Technic.—Necropsies were performed as soon after death as possible. Culture of the right heart's blood was made on pneumococcus broth. Material from all foci of infection was smeared and cultured. The smears were stained

[1] J. Exper. Med., 1926, 43, p. 573.

[2] J. M. Research, 1913, 29, p. 291.

[3] Ibid., 1916, 35, p. 151.

by Gram's and Hiss' capsule methods respectively. Cultures were made on hemolyzed sheep's blood agar plates and occasionally on rabbit's blood agar plates. Sections of lung and other tissues showing lesions were fixed in Zenker's and examined histologically.

PNEUMOCOCCUS INFECTIONS

There were 36 cases in which the pneumococcus was the sole or chief organism isolated from the pathologic lesions. Thirteen of these were males and 23 females, of which 14 were breeders. This much greater percentage of deaths in females, and especially among the breeders (which numbered only some 60 or 70), cannot be explained on the basis of a greater number of female than male animals in the stock used, as this was approximately the same.

TABLE 1

To Show Relative Mortality Incidence of the Various Infections

	Deaths	
	---	---
Organism	Number	%
B. Friedländer	56	49.2
Pneumococcus	36	31.6
Pseudotuberculosis (Eberthella)*	3	2.6
Bronchisepticus	5	4.4
Pneumonia of undetermined etiology	2	1.7
Streptococcus	3	2.6
Paratyphosus	1	0.9
Undetermined	8	7.0
	114	

* There were 11 other cases of this infection in which, however, the cause of death was attributed rather to the coexisting acute pneumococcus of B. Friedländer infection, with which they are therefore included.

Selter[4] reported a small epidemic in rabbits and guinea-pigs in two consecutive winters. He quotes similar reports by Weber and Stephansky. In 1919 Theobald Smith[2] described thirty-six cases of pneumonia in guinea-pigs. Seven of these were associated with the pneumococcus alone, while in four others it was isolated along with B. bronchisepticus. He also found pneumococcus infections in two other stocks and concludes that this organism must be regarded as a widely disseminated pathogenic organism among guinea-pigs. He quotes Binaghi, Ungermann, Christiansen and Richters as describing epizootics or isolating Gram positive diplococci from guinea-pigs. Stillman,[4a] at the Rockefeller Institute, has had four or five guinea-pig strains sent to him from outside sources, all of which were type 2 C, while Gardner,[4a] at the Saranac Lake Laboratory, frequently finds pneumococcus infections complicating his dust experiments. His strains are group 4. Holman[2] encountered the organism only three times. He quotes epidemics reported by Tartakowsky, Wittneben, Solomon and Kaspar and Kern, while Boni, like Selter, found these organisms in normal guinea-pigs.

[4] Ztschr. f. Hyg. u. Infektionskrankh., 1906, 54, p. 347.
[4a] Personal communications.

The Pneumococcus.—The organisms were all isolated at necropsy. They were from various sources: the heart blood, lung, pleura, middle ear, frontal sinus, pericardium, peritoneum and skin. They are gram-positive, lancet-shaped, capsulated, bile soluble, and ferment inulin. The final P_H when grown on 7.8 broth is 6.6 to 6.4. They are not agglutinated with the standard type 1, 2 and 3 serums. A rabbit anti-serum prepared from strain 68 agglutinated the homologous organisms in a dilution of 1 : 160 and also 6 other strains which were tested in 1 : 40 dilution. The virulence of the organisms is relatively low. Mice are killed regularly on intraperitoneal injection of the 1 : 100 dilution of an 18 hour broth culture. The dilutions above this are uncertain, the 1 : 1000 sometimes killing in a week. Small guinea-pigs sometimes succumb within a week to the 1 : 10 dilution, and ¼ cc. or over, intra-peritoneally, kill regularly.

Relationship of the Pneumococcus to the Human Type.—Theobald Smith suggested that it would be interesting to test the relationship of the guinea-pig pneumococcus to the human type. We have attempted to do this in two ways; by preparing the protein fraction of the guinea-pig pneumococcus and testing it against a pneumococcus protein anti-serum obtained from Avery at the Rockefeller Institute; and by agglutination tests using human group 4 strains and an antiserum prepared from a guinea-pig strain. Two protein solutions were prepared, one by breaking up the organisms in bile and the other by freezing and thawing. Both these preparations gave precipitin reactions against the pneumococcus protein antiserum. On the other hand, three human group 4 virulent strains obtained from the Rockefeller Institute tested against the guinea-pig pneumococcus antiserum failed to agglutinate.

Necropsy Findings.—The pneumococcus was isolated from the lesions in pure culture or was the predominant organism.

Septicemia occurred in thirty cases or 83.3%. In only one case was there no focus of infection found in the body and this was in a postpartum animal.

Pneumonia was present in eighteen, or 50% of all cases. Acute lobar pneumonia occurred in only one case, the whole right lung being involved. B. bronchisepticus was also isolated in this case. The remainder were all chronic processes. A characteristic lesion occurred in eight instances; this consists of multiple, greyish yellow, necrotic foci of varying size scattered over the surface of a dark purplish brown lung. All lobes, including the caudal, may be affected. Microscopically, these wedge-shaped areas resemble infarcts, have irregular edges and the base of the triangle is a thickened pleura. They are essentially necrotic tissue surrounded by a zone of polymorphonuclear leukocytes and fibrous tissue of greater or lesser thickness. In the remaining nine cases the chronic form of consolidation was not typical, but was merely a patch of dark purple, leathery tissue situated at the tip of one or more lobes

and usually small and multiple. Microscopically, congestion, atelectasis, irregular fibrosis and patchy pneumonia were seen. B. bronchisepticus was recovered from the lung along with pneumococcus in only three instances including the lobar lesion described above.

Exudative pleurisy occurred twenty-one times, or in 58% of all cases. It was coexistent with pneumonia in ten cases. A hemorrhagic fluid is characteristic and occurred in thirteen instances. One or both pleural cavities contain dark, bloody fluid and clots of blood enclosed in thin walled fibrous sacs. The exudate was serous in six cases and fibrinopurulent in one. Fibrous pleuritis occurred in three other instances.

Acute peritonitis was present in fourteen instances, or 39%. The amount of fluid varied from an unmeasurably small amount to 12 cc. The character of the fluid was serofibrinous or seropurulent in eight, hemorrhagic in three and fibrinopurulent in three. It occurred in both males and females and existed with or without pleurisy and pneumonia.

Pericarditis occurred ten times, or 28%. The exudate was hemorrhagic in five, serofibrinous in two, fibrinous in two (typical bread and butter heart), and purulent in one.

Otitis media occurred in eleven cases, or 30%. Both middle ears were involved in five cases and the right alone in six. The character of the exudate was serous in six instances, yellow pus in four, and inspissated and dry in one. Not infrequently the lining of the cavity was definitely red and congested. The petrous bone was thickened in three cases. Otitis associated with B. Friedländer occurred in five other cases.

The frontal sinuses were deeply congested, covered with exudate from which pneumococci were recovered in smear and culture in four cases. In ten other instances there was an associated B. Friedländer.

Cellulitis—extensive inflammation of the skin of the abdomen, with reddening, thickening and edema, was present twice.

The spleen in our series, excluding the seven cases with complications, was definitely enlarged in fifteen, 51.7%, of uncomplicated cases. The enlargement varied from slight increase to an organ estimated at six to eight times larger than usual. Many authors pay particular attention to this finding.

Two cases were complicated by generalized tuberculosis and five with multiple abscesses in the mesentery, liver and spleen, due to an organism of the Eberthella group described below. B. Friedländer sinusitis and otitis coexisted in five instances and sinusitis in ten others.

It will be seen from the above that hemorrhagic serous effusions are quite common in pneumococcus infections in guinea-pigs, especially hemorrhagic pleurisy. There is also a type of chronic consolidation of the lung which seems characteristic.

Analysis of the animals shows that only 5 belonged to the new stock, the remainder being all old stock. The infection occurred simultaneously throughout all the stock in different rooms and different cages. The epidemic ceased early in June. Whether this was due to the addition of grass to the food, to warmer weather, to the possibility that the susceptible animals had all died out, to a change in the character of the organism, or to vaccination of the younger stock and breeders, cannot be settled now.

Friedländer Bacillus Infections

There were 56 deaths attributed to this organism. Twenty-seven of the affected animals were males and 29 were females, including 11 breeders.

Direct allusions in the literature to infections of animals with the capsulatus group are meager. Friendländer[5] himself sprayed mice with human strains and produced pneumonia. R. Pfeiffer[6] in 1889 described the first case in guinea-pigs (B. Capsulatus Pfeiffer). Klein[7] working with B. pneumosepticus described an organism which is possibly in this group. Weaver[8] in 1898 found probably a B. mucosus capsulatus, and Smith[9] in 1894 isolated B. lactis-aerogenes once. Perkins[10] in 1901 described an epidemic in guinea pigs associated with B. mucosus capsulatus in which the characteristic feature was a gaseous emphysema of the liver and spleen. Holman[3] in 1916 found B. lactis-aerogenes in forty-eight animals and B. acidilactici in two. Endometritis occurred most frequently. Avery and Heidelberger[11] used a guinea-pig strain in their work on the soluble specific substance of B. Friedländer and Julianelle[12] used such a strain in typing organisms of this group. Gardner at the Saranac Lake Laboratory, Petroff at the Trudeau Laboratory and Stillman at the Rockefeller Institute are familiar with its occurrence in guinea-pigs. Stillman and Branch[13] used a guinea-pig strain in their work on experimental B. Friedländer pneumonia in mice. Jensen[14] found a capsulated organism in the normal intestine (B. centropunctatus). Chrom[15] thinks guinea-pigs particularly susceptible to B. Friedländer. Sisson and Walker[16] were able to infect cats experimentally. Chester[17] studied a case of pneumonia in a calf associated with B. mucosus capsulatus.

The Organism.—The strains were obtained at necropsy from many sources, i. e., the heart blood, lung, pleura, pericardium, peritoneum, middle ear and sinus. The organisms are large, gram-negative, plump rods, which vary in length and have a definite capsule. They are nonmotile, grow readily on broth, forming a heavy pellicle and diffuse turbidity. The agar colonies are highly refractile, sticky and are the color of pale gelatin. All the strains are gas formers, dextrose, saccharose, mannitol, levulose, maltose, salicin, galactose, arabinose and xylose being fermented, while lactose, dulcitol and adonitol are not fermented. Gelatin is not liquified nor is indol formed; nitrates are reduced and H_2S is formed.

Serologic Reactions.—The organisms agglutinate rapidly in type 2 pneumococcus serum and also in type B Friedländer bacillus serum obtained from Julianelle at the Rockefeller Institute, so that they undoubtedly fall into type B of the Friedländer organisms.

[5] Friedländer: Virchow's Arcb., 1882, 87, p. 319.

[6] Pfeiffer, R.: Ztschr. f. Hyg., 1889, 6, p. 145.

[7] Centralbl. f. Bakteriol., 1, O., 1889, 5, p. 625; ibid., 1891, 10, pp. 619, 841; ibid., 26, p. 260; Twenty-Second Report Local Government Board, 1892-3, 367; Centralbl. f. Bakteriol., 1. O., 1905, 38, p. 392. (Quoted by Holman.)

[8] Trans. Chicago Path. Sc., 1897-99, 3, p. 228. (Quoted by Holman.)

[9] Centralbl. f. Bakteriol., 1, O., 1894, 16, p. 237; J. M. Research, 1905, 13, p. 341; ibid., 1913, 29, p. 291. (Quoted by Holman.)

[10] J. Exper. Med., 1900, 5, p. 389.

[11] J. Exper. Med., 1925, 42, p. 701.

[12] Ibid., 1926, 44, pp. 113, 683.

[13] Ibid., 1925, 41, p. 623.

[14] Manual of Bacteriology, 1909, p. 225 (Quoted by Holman).

[15] Centralbl. f. Bakteriol., 1, O., 1911, 59, p. 103. (Quoted by Holman.)

[16] J. Exper. Med., 1915, 22, p. 747.

[17] Twelfth Annual Report of the Delaware College Agric. Exper. Sta., 1900.

Virulence: Mice, inoculated intraperitoneally, are rapidly killed with a 0.000001 dilution, the highest used. Small guinea-pigs succumb rapidly in the 1:10 dilution of an eighteen hour broth culture. Twelve guinea-pigs from an outside stock, which, by nasal swab examination, were first shown not to be Friedländer bacillus carriers, on three occasions had ¼ cc. of an eighteen hour culture run into either nostril. Two of these animals succumbed with acute B. Friedländer pneumonia and septicemia in five to six days respectively. Animals from our own stock are refractile to inhalation and seldom become infected.

Formation of an Avirulent Strain: Julianelle[12] has obtained several avirulent Friedländer strains by growing the organisms on the antiserum, as has previously been shown with pneumococcus (Reimann[18]). We were able to obtain an avirulent strain by the method used by Webster[1] in producing avirulent strains of B. lepisepticus. This organism gave the same sugar reactions as the type strain but failed to agglutinate in type serum. The method is as follows: The pellicle from a four day growth on lactose broth was removed, diluted and plated. After forty-eight hours' growth a colony differing from the characteristic colony was removed and subcultivated on lactose broth for four days. This was again replated and the resulting colony was smaller than the characteristic colony, not refractile or sticky and could be pushed along the surface of the agar plate without adhering. The growth on fluid media is very flocculent and granular. This organism kills mice in 0.25 cc. doses intraperitoneally but not in higher dilutions.

Necropsy Findings.—B. Friedländer was isolated from all the lesions.

Septicemia occurred in thirty-five of the fifty-six cases or 62.5%. It was most frequently associated with local lesions in the respiratory tract and external ear, i. e., sinusitis, pneumonia and otitis. The point of entrance in five cases was apparently the skin (cellulitis in three and abscess in two).

Sinusitis was most frequent, occurring in forty-five cases, or 80%. The turbinates were congested to a varying degree and exudate was present in varying amounts in all cases. In the majority it was sticky, mucoid material or greyish mucosus. In four animals necrosis of the turbinates had taken place. Otitis was a coincident lesion thirty-one times and pneumonia twenty-four times.

The middle ears were involved in thirty-seven of the animals or 66%. This was the second most common lesion. It was bilateral in twenty-nine cases and unilateral in eight, the left ear being affected alone in five and the right alone in three. The petrous part of the temporal bone was soft and necrotic in eleven cases and definitely thickened and resistant to the scissors in eight. The exudate was usually a thick, creamy, sticky pus. In some instances the external auditory canal contained dirty dried pus. Sinusitis was present in all but five cases and septicemia in nineteen.

Extradural abscess was a complication of ten of the otitis cases in which necrosis of the petrous portion of the temporal bone had occurred. In every case the creamy pus collected above the tentorium and encroached upon the temporal lobe, depressing it 1 to 2 cm. It occurred on the right side in seven instances, on the left in three and was bilateral in one. Septicemia was present in only two cases.

Abscess under the scalp occurred in five of the above eight cases of extradural abscess. The pus had burrowed through the parietal bone, which was either necrotic or showed a large orifice and formed a well recognizable, soft, fluctuat-

18 J. Exper. Med., 1925, 41, p. 587.

ing mass on the head under the scalp. The right parietal bone was eroded in three cases, and the left parietal in two. In no case was septicemia present.

Pneumonia as a definite gross and microscopic consolidation of the lung, occurred in twenty-six animals or 46%. In seven animals, three of which were young, there was typical acute B. Friedländer pneumonia. The lesion was lobar in distribution and one or more lobes were affected. In the remaining nineteen cases, both histologically and in the gross, the lesions were old and chronic, usually multiple and involving the anterior or upper tip of the cephalic and ventral lobes and the medial lobes. The consolidation was usually greyish red in color and gelatinous with slight corrugation of the surface, but often it was red and beefy and once cartilaginous. Sinusitis was a coexisting lesion in all but two cases and septicemia occurred in nineteen, six of which were the acute pneumonias.

Pleuritis was present in fourteen cases or 20%, ten times with pneumonia, four of which were in the acute consolidation. It was bilateral in twelve cases and occurred on the left side alone in two. The effusion was serous in three, bloody in one, fibrinopurulent in nine, and in one a localized encysted pocket of pus was found. Septicemia was present in twelve cases.

Pericarditis occurred in six instances, all with septicemia and pneumonia. The exudate in the pericardial cavity was mucoid in four, serofibrinous in one, and fibrinous and hemorrhagic in one.

Peritonitis occurred six times. In every case there was septicemia. The exudate was fibrinopurulent in three, serous in two and serofibrinous in one.

A diffuse, widespread cellulitis of the abdomen and side occurred in three cases. In all of these there were abrasions of the skin, probably bites, and septicemia was concomitant.

Skin abscess with a pocket of thick, yellow, flocculent pus was found in four cases, in two of which there was septicemia and inflammation of one or more serous cavities.

Metritis occurred in two cases. The uterus was large and red, the wall was thickened and filled with pus. The mucosa was very injected.

Complications: Generalized tuberculosis was present in one case and pseudo-tuberculosis in seven. In two cases of otitis the pneumococcus was isolated and in one B. bronchisepticus. B. bronchisepticus was also recovered from a pneumonic patch in one instance.

Microscopic Examination of the Lungs.—Acute Cases: These were all typical Friedländer bacillus acute pneumonias such as have been produced experimentally (Stillman[13]).

Chronic Cases: No typical histologic picture occurred. The lungs were usually atelectatic and the alveolar walls sometimes congested. Mononuclear cells occupied the alveoli in varying degrees and more or less widespread fibrosis and fatty degeneration were to be seen. Adenomatous-like formations around the bronchi were not uncommon. Occasionally small abscesses occurred.

Table 2 shows the relative frequency of the various lesions associated with B. Friedländer and with pneumococcus. It will be noted that sinusitis and otitis are more commonly associated with B. Friedländer, whereas generalized and acute inflammations of serous cavities are more often associated with the pneumococcus.

eberthella caviae (pseudotuberculosis)

Fourteen animals died with lesions of pseudotuberculosis, from 12 of which an organism of the Eberthella group (Bergey's [19] classification) was isolated. Twelve were females, seven being from the breeding cages. The infection was localized to five cages in two rooms.

Many organisms have been described as associated with the lesions of pseudotuberculosis but the organism here described cannot be absolutely identified with any one of them, though it most closely resembles the organism originally described by Pfeiffer.

The literature is reviewed from the point of view of etiology.

B. Paratyphosus Group: These are perhaps the organisms which produce most characteristically multiple abscesses in rodents. They include B. paratyphosus, B. enteritidis (Gaertner), B. aertrycke, and B. paratyphosus A (Kirch). The earlier descriptions [20] are inadequate for actual classification of

TABLE 2

The Relative Incidence of the Various Lesions

	Septi-cemia	Pneu-monia	Pleurisy	Peri-tonitis	Peri-carditis	Otitis	Sinu-sitis	Extra-dural Abscess	Cellu-litis
With Pneumococcus Infections									
Number of animals....... (Total, 36; 23 females)	30	18	21	14	10	11	4	0	2
Percentage..............	83	50	58	39	28	30	11	0	5.6
With Friedländer's Bacillus Infections									
Number of animals....... (Total, 56; 29 females)	35	26	14	6	6	37	45	10	3
Percentage..............	62.5	46	20	10.7	10.7	66	80	17.8	5.3

the organisms. Theobald Smith [9] in 1894 produced the first evidence and later proved the point with B. suisepticus. Durham [21] produced the lesions experimentally with B. enteritidis (Gaertner) by feeding and this was done later by Dieterlen and Kirch. Others [22] also report epizootics. Wherry [23] described B. pestis caviae and Petrie and O'Brien [24] lost all but twenty-one of 500 animals with B. aertrycke infection. Bainbridge and O'Brien [25] found abscesses in the mesenteric glands. B. caseolyticus (Lochmann [26]), a variety of B. coli thought identical with B. caseolyticus (Kovarzik [27]) and B. pseudotuberculare orchitophlogenes (Cagnetto [28]), probably also fall into this group.

[19] Manual of Determinative Bacteriology, 1926.
References 20 to 28, inclusive, and 30, 35, 36, 37 are quoted by Holman.
[20] Malassez and Vignal: Kolle and Wassermann, Handbuch der pathogenen Mikroorganismen, 1912, 5, p. 776; Chantemesse: Ann. de l'Inst. Pasteur, 1887, 1, p. 97; Charrin and Rogers: Centralbl. f. Bakteriol., 1, O., p. 1888, 4, p. 44; Dor: Compt. rend. de l'Acad. sci., 1888, 106, p. 1027.
[21] Brit. M. J., 1898, 2, p. 600; ibid., 1899, 1, p. 1216.
[22] Dieterlen: Centralbl. f. Bakteriol., 1, Ref., 1909, 44, p. 282; MacConkey: J. Hyg., 1905, 5, p. 333; de Basi: Centralbl. f. Bakteriol., 1. Ref., 1909, 44, p. 86; Eckersdorf: ibid., 1906, 38, p. 99.
[23] J. Infect. Dis., 1908, 5, p. 519.
[24] J. Hyg., 1910, 10, p. 287.
[25] J. Path. & Bact., 1911, 16, p. 145.
[26] Centralbl. f. Bakteriol., 1, O., 1902, 31, p. 385.
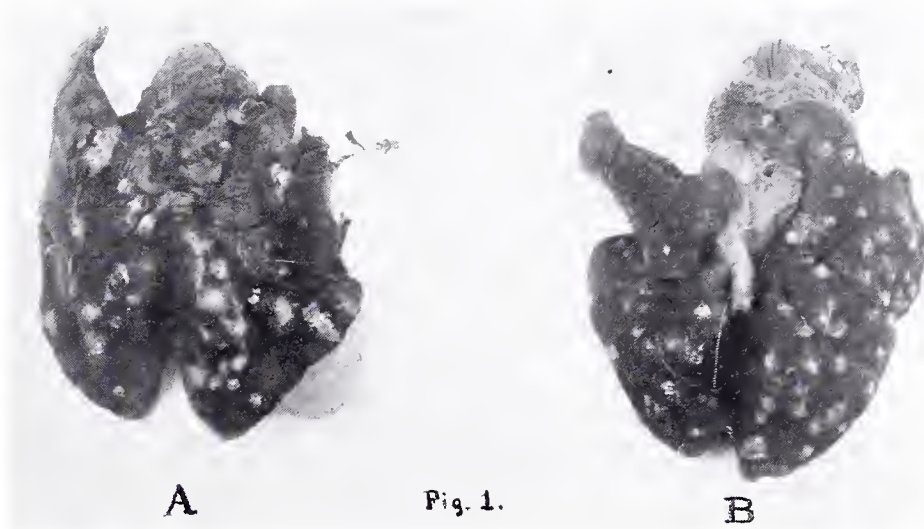[27] Ibid., 33, p. 143.
[28] Ann. de l'Inst. Pasteur, 1905, 19, p. 449.

Fig. 1.—(A) Photograph of guinea-pig's lungs showing the lesions of Pseudotuberculosis (Eberthella caviae). (B) Photograph of guinea-pig's lungs to show for comparison tuberculosis of the lung (Mycobacterium tuberculosis humanis) occurring in generalized experimental tuberculosis.
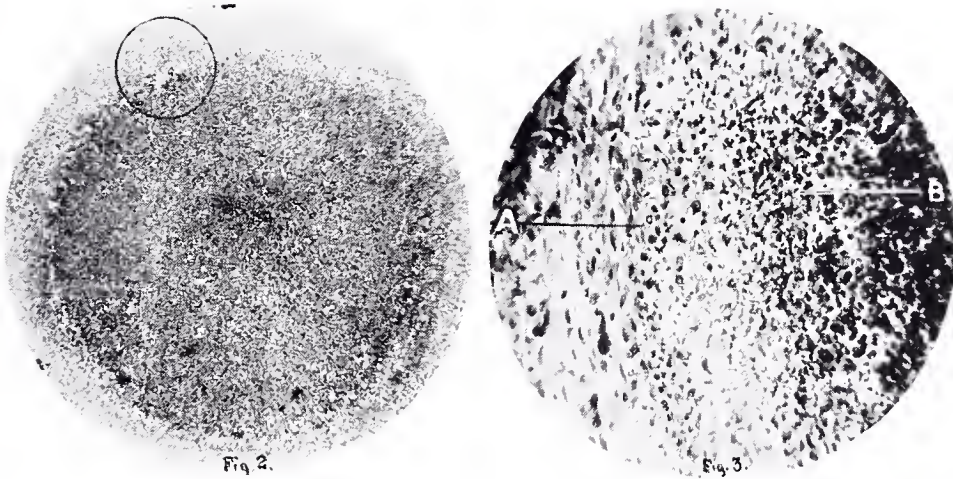

Fig. 2.—Microphotograph of a pseudotubercle in the liver of a guinea-pig. Low magnification. Circle: portion shown in fig. 3.

Fig. 3.—Microphotograph of the periphery of the nodule seen in figure 2. A: the interior of the nodule composed of a mass of cells. B: the fibrous wall. Higher magnification.

Streptococci: They have been described by Chantemesse, Dor,[20] Boxmeyer,[29] and others.[30] Boxmeyer's [29] epidemic was quite an extensive one and more than 100 necropsies were done. The cervical, submental and auricular glands were affected in 90% of the cases, the others less frequently. In a few cases an organism of the hog cholera group was isolated as well as streptococci. He produced the disease experimentally by feeding the hemolytic streptococcus isolated. Pasteurella group: Byloff [31] in 1906 described a typical pseudotuberculosis in guinea-pigs. He isolated an organism which he classified in the hemorrhagic septicemia group and produced the disease experimentally (B. pestis intestinalis caviae cob.). He considered it the same as an organism previously described by Zalatogoroff [32] and A. Pfeiffer [33] in spite of its motility. Dieter and Rhodes [34] have encountered lesions in guinea-pigs which resemble plaque and are due to bipolar organisms not culturally B. pestis. B. Coli: An organism of this group is described,[35] producing either pseudotuberculosis or abscess. Micrococcus tetragenous: Kasper and Kern [36] describe numerous abscesses associated with this organism mixed in two cases with a gram-negative and in one with a pneumococcus. Spore formers: Eberth and Dor recovered these from cases of pseudotuberculosis. Coccidea: Cases of coccidiosis are described.[37] Natural plague: Holman [3] points out that this occurs spontaneously in India. Tularemia: Dieter and Rhodes [34] have recently reported lesions in guinea-pigs produced by injecting material from wild oats suspected of carrying B. pestis, which resembled the lesions of experimental plague but were due to B. tularense. B. abortus: Schroeder in Washington showed Petroff lesions caused by this organism in guinea-pigs and he was struck with the superficial resemblance to tuberculosis. Blastomycosis: Long [37a] states that injection of this organism causes in guinea-pigs multiple abscesses not unlike tuberculosis. Rivas,[38] mentions that blastomycosis in animals may be produced by another genus of Saccharomyces, i. e., the cryptococcus. Diphtheroid group: Several authors [39] have described the occurrence of the corynebacterium rodentium in pseudotuberculosis of rodents.

Gross Pathologic Findings.—The mesenteric glands were involved in every case. The glands varied in size from an almond to a fused mass the size of a full term guinea-pig fetus. The gland at the junction of the cecum and ileum was always involved but usually not greatly enlarged. The glands were completely transformed into a mass of thick, putty-like, yellowish white pus and fibrous tissue. The capsule showed great fibrous thickening, the gland appearing pearly white. The liver and spleen were affected in ten instances, 71%. The spleen was enlarged, sometimes six to eight times the normal size. The surfaces

[29] J. Infect. Dis., 1907, 4, p. 657.
[30] Eberth: Virchow's Arch., 1885, 100, p. 15; Lamar: J. Exper. Med., 1909, 11, p. 152; Horne: Centralbl. f. Bakteriol., 1, O., 1912, 66, p. 169.
[31] Centralbl. f. Bakteriol., 1, O., 1906, 41, pp. 707, 787; 42, p. 5.
[32] Ibid., 1904, 37, p. 654.
[33] Ueber die Bacilläre Pseudotuberkulose bei Nagatieren, 1889.
[34] J. Infect. Dis., 1926, 38, p. 541.
[35] Galli-Valerio: Centralbl. f. Bakteriol., 1, Ref., 1900, 27, p. 305; Schwartz: Ibid., 1, O., 1906, 36, p. 273; Simon: Compt. rend. Soc. de biol., 1910, 69, p. 393.
[36] Centralbl. f. Bakteriol. 1, O., 1912, 63, p. 7.
[37] Strada and Traina: Centralbl. f. Bakteriol., 1, O., 1900, 28, p. 635; Pianese: Ztschr. f. Hyg., 1901, 36, p. 350; Griffith: Report Royal Comm. Tuberculosis, 1907, 1, pt. 2.
[37a] Personal communication.
[38] Textbook on Human Parasitology, 1920, p. 594.
[39] Pfeiffer: Ueber die Bacillare Pseudotuberkulose bei Nagatieren, 1889; Preisz: Ann. de l'Inst. Pasteur, 1894, 8, p. 231; Migula: System der Bakterien, 1900, p. 374. Cited from Bergey,[19] p. 386.

of both organs were studded with small round white nodules. These were apt to be discrete and varied in diameter from 1 to 4 mm. The edges were well outlined from the surrounding tissue and in some cases the abscesses were slightly but definitely raised above the surface. Each nodule was filled with pus similar to that found in the mesenteric glands. The intestine showed a few small abscesses in eight cases. These were practically always in the ascending colon, less often in the cecum and occasionally in the small gut. The lung showed numerous small scattered yellow abscesses surrounded by a narrow zone of congestion in seven cases. The omentum was involved in five cases. The organ was rolled up around the inferior surface of the stomach and showed numerous small abscesses.

TABLE 3

PSEUDOTUBERCULOSIS

| Guinea-pigs | | | | Organs Affected | | | | | | Complications |
| | | | | Mesenteric | | | Omen- | | | |
No.	Sex	Cage	Organism	Glands	Liver	Spleen	tum	Lungs	Gut	
1	Female	B 1	Eberthella	+	+	+	0	+	+	Friedländer's otitis and sinusitis
2	Female	B 1	Friedländer	+	0	+	+	0	0	Friedländer's septicemia
3	Female (breeder)	A 3	Eberthella	+	+	+	+	0	+	Friedländer's sinusitis, pneumococcus otitis
4	Female	B 1	Friedländer	+	+	+	0	0	+	Friedländer's septicemia and pneumonia
5	Female (breeder)	A 2	Eberthella	+	+	+	0	+	+	
6	Female (breeder)	A 2	Eberthella	+	+	+	+	0	0	
7	Female (breeder)	A 3	Eberthella	+	0	0	0	0	+	
8	Female (breeder)	A 3	Eberthella	+	0	0	0	0	+	Pneumococcus septicemia
9	Male	B 8	Eberthella	+	+	+	+	+	+	Friedländer's septicemia
10	Female	B 1	Eberthella	+	0	+	0	+	0	Pneumococcus septicemia
11	Female (breeder)	A 2	Eberthella	+	+	+	0	+	0	Friedländer's otitis
12	Male	B 2	Eberthella	+	+	0	0	+	0	Pneumococcus septicemia
13	Female (breeder)	A 3	Eberthella	+	+	0	0	0	0	Pneumococcus septicemia
14	Female	B 1	Eberthella	+	+	+	+	+	+	Friedländer's septicemia
Total number (Eberthella 12) (Friedländer's 2) 14				14	10	10	5	7	8	
%				100	71	71	36	50	57	

Microscopic Examination.—The nodules are sharply outlined from the surrounding liver structure by a well developed layer of fibrous tissue. In the liver the contiguous layer of liver tissue shows degeneration, and a fatty metamorphosis of the whole liver is quite frequent. The interior of the nodule is composed of a mass of cells and nuclear debris. Most of the cells are mononuclears but there are polymorphonuclears, particularly around the periphery. In the lung the lesions are not as well walled off by a fibrous capsule. In the gut the abscesses are situated in the submucosa. The caseous glands are really composed of a series of small abscesses surrounded by dense fibrous tissue which fuse in the later stages. In the spleen they appear to occur chiefly in Malphigian corpuscles and the pulp is atrophic. The omental lesions are in the substance of the organ.

The Organism.—As stated above an organism of the Eberthella group was isolated in twelve of fourteen cases. In the other two cases B. Friedländer was recovered. Both were, however, complicated by B. Friedländer septicemia, so that probably technical difficulties account for the failure to isolate the Eberthella organism.

The organism as isolated from the abscesses in various organs is a plump, gram-negative, nonmotile bacillus, measuring in broth cultures 0.5 x 0.8 to 2 microns. On gelatin coccoid forms are more common, whereas on agar with 5% sodium chloride longer rods than the above are found. Involution forms occur in old cultures. No polar staining, no spores and no capsules were demonstrated. In the direct smear of the pus from one of the abscesses, it is extremely difficult to find organisms. They are few in number and in some instances we failed to find any. No acid fast organisms were ever demonstrated. They grow well aerobically. On broth a heavy sediment forms with a slight pellicle. On agar the colonies in twenty-four hours are less than 1 mm. in size and the edges are somewhat rough, the outline being irregularly octagonal. Maximum growth occurs in forty-eight hours when the individual colonies are 1.5 to 2 mm. in size. They are not strictly confluent though they may be in apposition. The colonies are translucent and bluish grey, resembling the colonies of B. bronchisepticus at first. They are convex, the center being distinctly raised but not granular. They do not adhere to the needle, nor are they very sticky. Gelatin is not liquified and the growth here is heavy, arborescent on the surface and with fine, delicate, coral-like offshoots in the deeper parts. The general characteristics are summarized as follows:

EBERTHELLA CAVIAE: BIOLOGIC AND CULTURAL CHARACTERISTICS

Gram-negative	Voges-Proskauer reaction negative
No polar staining	Indol not formed
Size: 0.5 by 0.8 to 2.0 microns	Nitrates reduced
Nonmotile	Hydrogen sulphide
No capsules, nor spores	Litmus milk, alkaline
Broth: sediment and pellicle	
Agar: translucent colonies 2 mm.	
Gelatin: arborescent, no liquefaction	

CARBOHYDRATE REACTIONS

Acid produced in	No fermentation in
Dextrose, galactose, levulose, maltose, mannose, xylose, mannitol, dulcitol, salicin	Saccharose, lactose, arabinose, adonitol, dextrin
Agglutination negative with antiserums for B. typhosus, B. paratyphosus A and B, B. dysenteriae (Flexner and Hiss)	Virulent for mice and guinea-pigs

These findings were corroborated by L. T. Webster, by Holman, and by Bergey who says that in the absence of polar staining the organism undoubtedly falls into the Eberthella group. He adds the following additional characteristics: Russell's double sugar agar shows acid base but no gas. There is no action on lead acetate in this medium. Tests with immune serums showed no agglutination with antityphoid, antiparatyphoid A and B, antidysentery or antiparadysentery (Flexner and Hiss) serums. Holman thinks the organism comes closer to B. pseudotuberculosis rodentium (Pfeiffer) than to any other.

In view of the fact that this organism shows carbohydrate fermentations like some strains of Friedländer bacillus and as infection with B. Friedländer has been common in our stock, it seemed necessary to exclude the possibility of its being an avirulent B. Friedländer organism.

This we think we have done by agglutination tests. The organisms were cross agglutinated against two antiserums obtained from the Rockefeller Institute prepared by using avirulent Friedländer organisms. The results were negative. Similarly, agglutination tests using a rabbit antiserum made from a strain of these organisms against two avirulent B. Friedländer strains (one of ours and one of Dr. Julianelle's) were negative. Further evidence against it being an avirulent B. Friedländer appears below, since, unlike B. Friedländer strains, they produce lesions in guinea-pigs.

Virulence: Mice inoculated intraperitoneally with a 24 hour broth culture are killed with 0.25 cc. and over. Small guinea-pigs inoculated intraperitoneally with 0.25 cc. die in three to four days with serous peritonitis and abscesses in the liver, spleen and omentum. Subcutaneously death ensues after a similar dose in 10 days. The local glands show caseation; there is marked reaction sometimes with necrosis at the site of inoculation and abscesses in the liver and spleen more numerous.

Experimental Infection by Mouth.—One cc. of a 24 hour broth culture was fed by a stomach tube to a full grown female. In five weeks the animal was killed and showed no pathologic lesions.

At the same time, using the same culture and similar procedure the tube was introduced into the posterior pharynx and some regurgitated through the mouth. This animal died in three weeks and the necropsy findings follow:

In the neck are four large lymph glands, measuring 1 cm. in diameter, filled with caseous pus. The mesenteric glands are slightly enlarged but grossly normal. The hepatic gland shows many small caseous foci. There are 3 cc. of clear serous fluid in the abdomen. The spleen is enlarged to twice its normal size. There are many pin-head sized abscesses and a few 3 mm. in diameter on the surface and in the deep splenic tissue. These contain thick, putty-like, yellowish white pus. The liver is studded with small abscesses. The omentum is rolled up in the upper abdomen and shows many small abscesses. There are a few abscesses in the meso-colon and on the parietal peritoneum of the lower left abdominal wall. There are many small, round, yellowish foci in the lungs around which are areas of congestion. The heart blood culture was negative. A few, short, plump, gram-negative rods were seen in the direct smear from the abscesses in the cervical glands, the liver and spleen. In the neck glands there were also a few cocci. No acidfast bacilli were demonstrated. The Eberthella organism was cultivated from the cervical glands and also gram-positive cocci.

The pus from one of the cervical lymph glands was dissolved in saline and 1 cc. inoculated into 2 guinea-pigs, one receiving 1 cc. of the emulsion intraperitoneally and the other 1 cc. subcutaneously. The first animal died in 6 days and showed some free peritoneal fluid and multiple abscesses in the spleen, which was enlarged, and in the liver and lung. Smears of the pus showed gram-negative rods and no acid-fast bacilli. The second animal which received the subcutaneous inoculation died in 9 days. There was an indurated necrotic focus surrounded by an area of congestion 1.5 cm. in diameter at the site of inoculation in the groin. Two of the local inguinal glands measured 7 mm. in diameter and were filled with caseous pus. The liver, spleen and lung showed multiple small abscesses. Smears of the pus showed gram-negative rods and gram-positive cocci. Four other animals inoculated with similar doses of two avirulent Friedländer strains remained well.

A third animal was also inoculated in the groin with caseous material from the cervical glands, the pus having been previously treated with N/1 NaOH for ½ hour in the incubator. The centrifugalized material was used for inoculation and cultured on egg medium and Petroff's medium. The cultures grew and the inoculated guinea-pigs developed enlarged glands locally palpable from the fourth day and growing larger until the animal was killed on the thirteenth day. At necropsy there were four large caseous inguinal glands in the left groin, the largest being 1.5 cm. in diameter. The left iliac gland was also greatly enlarged and caseous. The rest of the necropsy was negative.

Experimental Infection by Inhalation.—Three small male guinea-pigs were exposed in a spray chamber to a mist produced by an atomizer which contained an emulsion of an 18 hour agar growth of the organisms in saline. The first animal died in 10 days, the second in 12 and the third in 14 days. The animals appeared perfectly well until about 36 hours before death when they appeared sick. The necropsy findings in the lungs in all cases were identical.

There were loose fibrinous adhesions between the visceral and parietal pleura. The lungs showed many large greyish yellow areas scattered through all lobes. There were some 4 mm. in diameter with irregular borders and they were surrounded by areas of congestion. The bronchial lymph glands were large, congested and showed scattered caseous foci. The heart blood culture was positive (Eberthella caviae) in only one instance. In two of the animals there was no other finding but in the third, which lived longest, there were two abscesses in both the liver and spleen. The specific organism was cultured in every case from the local lesions and the smears were negative for acidfast bacilli. In the x-ray examination of one of the animals, the day before death, many shadows which suggested a bronchopneumonia were shown in the lungs.

Microscopically the lesions in the lungs of the experimentally infected animals resemble those seen in the spontaneously infected except that the lesion is rather more acute, more extensive and contains more organisms. It will thus be seen that the lesions develop much more rapidly than in the case of true tubercle.

Method of Infection in the Spontaneous Cases.—We have failed experimentally (by inoculations subcutaneously and intraperitoneally, by inhalation and by mouth) to reproduce the disease exactly as it occurred spontaneously. In view of the fact, however, that one of the animals in which a culture was introduced into the throat developed local caseous glands and multiple abscesses, we feel that in all probability the infection naturally occurs by way of the digestive tract or in females through the uterus, the mesenteric glands being thus first involved. The fact that all animals fed by mouth do not develop the disease might account for the absence of infection in other animals in the same cage and also for the small number of animals dying of the disease. The disease undoubtedly spreads by the lymphatics, the lymph glands early showing greater involvement than the site of inoculation.

Identification of the Organism.—The organism described as associated with pseudotuberculosis in the present epidemic belongs to either the Eberthella or the Pasteurella groups in Bergey's classification. The latter would seem the more plausible but in the absence of polar staining and involution forms and by the relatively large number of carbohydrates fermented, we feel justified in placing it, at least temporarily, in the Eberthella group, even though there is no cross agglutination with other members of this group. The growth on gelatin is somewhat arborescent and they are hardy organisms apparently withstanding treatment with sodium hydroxide.

RESUMÉ AND DISCUSSION

In our experience spontaneous infections are relatively common among guinea-pigs. B. Friedländer and pneumococcus, group 4, are the organisms most frequently isolated from the lesions in our own stock at Trudeau Sanatorium Laboratory, and not B. bronchisepticus or B. paratyphosus which are found more often in some localities. Besides the reports already cited certain others [39a] also describe such infections in guinea-pigs. The extreme chronicity of some of the lesions is of interest and importance as this chronic focus of infection may play some part in altering the resistance of the infected animal to experimental inoculation with tubercle bacilli and lead to false conclusions. Petroff and Stewart [40] have observed that animals which show extensive tuberculosis after inoculation rarely develop other infections (e. g., pneumonia), and the reverse may also hold. We have followed animals with snuffles and otitis clinically and with nasal swabbing over a period of months and find that at necropsy these lesions are the cause of death. Although the majority of these animals may show signs of disease, for instance, exudate in the nares, incoordination when the otitis is unilateral, or emaciation, many have been found without visible local signs of infection. This is so if the otitis is bilateral as it is most frequently. Many of the animals may also be extremely well nourished with sleek coats and persist in this state for eight months, the period over which our observations have extended. One would also stress the necessity of opening the sinuses and middle ears at necropsy in every case, as we have found a very high percentage of otitis and sinusitis in our necropsies. In about 6% of the cases the cause of death would otherwise not have been found while if blood cultures are not done routinely, this percentage would be considerably higher. Why the infections are more common in the winter and may acquire epidemic proportions we cannot say, but feel that possibly the absence of sufficient green vegetables may be partly responsible. The portal of entry in most cases appears to be by the upper respiratory tract, middle ear and uterus. That chronic pulmonary infections associated with B. Friedländer may occur in humans has recently been pointed out by Belk,[41] who found 3 in 18 cases of such infections. The clinical and pathologic findings were confused with tuberculosis.

[39a] Cotoni, Truche and Raphael: Pneumococcus and Pneumococcal Affections (Translation), 1924; Thomas: J. Infect. Dis., 1924, 35, p. 407; Howell, Katharine M., and Schultz, O. T.: J. Infect. Dis., 1922, 30, p. 516; Trawinski, A.: Centralbl. f. Bakteriol., 1, O., 1922, 88, p. 24.
[40] J Immunol., 1926, 12, p. 97.
[41] J. Infect. Dis., 1926, 38, p. 115.

From the cases of pseudotuberculosis we have recovered an organism which is probably in the Eberthella group (Bergey's classification). The resemblance of the lesions grossly to true tuberculosis renders it necessary to demonstrate acidfast organisms in doubtful cases, as in direct smears the organisms of pseudotuberculosis may be present in so few numbers as to escape detection.

SUMMARY

Fifty-six cases of B. Friedländer and 36 cases of pneumococcus group 4 infections in guinea-pigs are described, the lesions included septicemia, sinusitis, otitis, pneumonia, pleurisy, pericarditis, peritonitis, metritis, cellulitis and focal abscesses. The Fredländer organism belongs to type B in Julianelle's classification, i. e., that group which cross agglutinates with type 2 pneumococcus antiserum. The pneumococcus does not agglutinate with antiserums of any of the fixed types but is related serologically by its protein fraction with human strains.

The frequency of spontaneous chronic infections in guinea-pigs is stressed and the possible effect these may have on altering the resistance of the infected animal to subsequent experimental inoculations with tubercle bacilli, is discussed. The point of entry in the majority of instances appears to be by the upper respiratory tract and middle ear and the necessity of examining the sinuses and ears at necropsy is emphasized.

Fourteen cases of pseudotuberculosis are described. Twelve of these were associated with an organism of the Eberthella group (Bergey's classification) and some experimental evidence is afforded that it is the specific infective agent and that infection can occur by feeding.

Caution is advised in diagnosing tuberculosis by gross inspection alone.

RHEUMATIC FEVER

BACTERIOLOGIC STUDIES OF A NON-METHEMOGLOBIN-FORMING STREPTOCOCCUS WITH SPECIAL REFERENCE TO ITS SOLUBLE TOXIN PRODUCTION

Konrad E. Birkhaug

From the Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

Recent studies on the production of soluble toxins by Streptococcus scarlatinae,[1] Streptococcus erysipelatis,[2] and certain strains of Staphylococcus aureus,[3] suggested a study of possible toxin production by a series of other streptococci. The first indication of a toxin producing streptococcus associated with rheumatic fever was obtained by the author in March, 1926, when an unusual strain of a nonhemolytic and non-methemoglobin-forming streptococcus was repeatedly isolated from blood cultures taken from a 5 year old girl, admitted to the Strong Memorial Hospital, on March 23, 1926. The patient was found to be ill with acute rheumatic fever, endocarditis, myocarditis, pericarditis, and pleurisy. On March 31 the patient expired and at autopsy, the clinical diagnosis was confirmed. From the vegetations on the mitral valves a streptococcus was isolated, identical biologically with the organism obtained from blood cultures during life. At that particular time, our laboratory was engaged in the study of soluble toxins produced by streptococci associated with scarlet fever and erysipelas. The strains of non-methemoglobin-forming streptococci isolated from the case of rheumatic fever, labelled in these studies as RF. 1, were found to produce a potent, soluble, thermostable toxin, similar in many respects with those produced by the scarlet fever and erysipelas streptococci. Perhaps, more striking than their ability to produce a soluble toxin, was their action upon the complex carbohydrates, in that they consistently fermented inulin and salicin, and differed definitely from the pneumococcus in that they remained bile insoluble. The unusual biological character-

[1] Dick, G. F., and Dick, G. H.: J. Am. M. A., 1925, 84, p. 802; Northwest Med., 1926, 25, pp. 111, 200; Henry, H., and Lewis, F. C.: Lancet, 1925, 2, p. 587; Kirkbride, Mary B., and Wheeler, Mary W.: J. Immunol., 1926, 11, p. 477; Young, C. C., and Orr, P. F.: J. Am. M. A., 1926, 86, p. 1340.

[2] Birkhaug, K. E.: Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 201; Singer, H. A., and Kaplan, Bertha: Arch. Path. & Lab. Med., 1926, 2, p. 461; J. Am. M. A., 1926, 87, p. 2141.

[3] Parker, Julia E.: J. Exper. Med., 1924, 40, p. 761.

istics of these cultures isolated from a typical and fatal case of rheumatic fever aroused our interest and during the past year an earnest effort was made to determine the frequency with which such organisms were present in persons stigmatized by rheumatic fever and definite allied forms of this protean disease, and whether or not the production of a soluble toxin was a constant feature of such microorganisms. These preliminary studies are based essentially on experiments conducted with a group of streptococci, similar in many respects with the RF. 1 strain, and isolated from typical cases of rheumatic fever and its syndromes.

Similarity in symptoms and signs elicited during the acute attacks of rheumatic fever with those obtainable in septic sore throat, angina, tonsillitis, scarlet fever, and erysipelas, has suggested the presence of a circulating toxic substance during the acute and relapsing attacks of rheumatic fever, a disease known to commence with an attack of sore throat and tonsillitis. In a recent and careful statistical study of 317 cases of rheumatic fever, Mackie,[4] found that 80.6% gave a definite clinical history of focal infection of the upper respiratory tract, and a large group of investigators,[5] have for some time repeatedly pointed out the significance of such infectious foci in relation to the etiology of rheumatic fever and its allied conditions.

Extensive studies in the bacteriology of rheumatic fever, notably the invaluable immunological works by Swift and Kinsella,[6] and Andrews, Derick and Swift,[7] have demonstrated the infrequency with which streptococci are isolated from the blood stream of acute rheumatic fever patients (less than 10%). These authors have gone farther than others in their serological analysis of streptococci isolated from a variety of sources associated with rheumatic fever and their earnest efforts to demonstrate a specific type of streptococcus in rheumatic fever have failed consistently. In the absence of any indisputable specific virus to which one might ascribe the etiologic role in the production

[4] Mackie, T. T.: Am. J. M. Sc., 1926, 172, p. 199.

[5] Burbank, R., and Hadjopoulos, L. G.: J. Am. M. A., 1925, 84, p. 637; Coombs, C. F., and Poynton, F. J.: Brit. M. J., 1926, 2 (suppl.), p. 13; Crowe, S. J., Watkins, S. S., and Rothbolz, A S.: Bull. Johns Hopkins Hosp., 1917, 28, p. 1; Davis, D. J., and Pilot, I.: J. Infect. Dis., 1919, 24, p. 386; Davis, D. J.: J. Infect. Dis., 1921, 29, p. 524; Ill. M. J., 1919, 36, p. 134; J. Am. M. A., 1919, 72, p. 319; Gording, R.: Norsk Mag. f. Laegevidensk., 1925, 86, p. 995; Holsti, Ö.: Arbeiten aus dem Pathologisch-Anatomischen Institut. Helsingfors. 4, Nos. 3/4; Finska Läk. Sällsk. Handl., 1924, 66, p. 365; 1926, 68, p. 414; Hunt, G. R.: Guy's Hospital Reports, 1923, 73, p. 383; Ingerman, E., and Wilson, M. G.: J. Am. M. A., 1924, 82, p. 759; Kaiser, A. D.: Ibid., 1926, 87, p. 1012; King, J. J.: M. Clinics N. Am., 1917, 1, p. 799; J. Am. M. A., 1917, 69, p. 91; Lawrence, W. S.: J. Am. M. A., 1920, 75, p. 1035; Ibid., 1922, 79, p. 2051; Lewison, M.: Ibid., 1923, 80, p. 828; MacCallum, W. G.: Ibid., 1925, 84, p. 1545; Meyer, J., Pilot, I., and Pearlman, S. J.: J. Infect. Dis., 1921, 29, p. 59; Nordlund, H.: Hygiea, 1919, 81, p. 497; Pemberton, R.: Arch. Int. Med., 1920, 25, pp. 231, 241, 335; Poynton, Patterson and Spence: Lancet, 1920, 2, p. 1086; Schmidt, H. B.: J. Am. M. A., 1922, 79, p. 956; Singer, G.: Med. Klinik, 1925, 21, p. 1530; Starling, H. S.: Guy's Hospital Reports, 1923, 73, p. 388; Quart. J. Med., 1923, 16, p. 263; Swift, H. F.: Boston M. & S. J., 1922, 187, p. 331; Tongs, M. S.: J. Am. M. A., 1919, 73, p. 1050; Troisier, J.: Bull. et mem. Soc. med. d. hôp. de Paris, 1925, 49, p. 629; Tsuchiya, H.: Arch. Int. Med., 1925, 36, p. 636; Walker, J. E.: J. Infect. Dis., 1920, 27, p. 618; Wiesel, J.: Med. Klinik, 1923, 19, pp. 163, 197.

[6] Arch. Int. Med., 1917, 19, p. 381; Am. J. M. Sc., 1925, 170, p. 631.

[7] J. Exper. Med., 1926, 43, p. 13.

of rheumatic fever, it has of late become increasingly evident that the clinical and pathological picture in rheumatic fever is a constant and almost specific disease entity, and one that is invariably linked up with some part of the circulatory system. That the damage to specific tissues in the body may be the end result of a constant production and fixation of bacterial toxins, absorbed from foci such as infected tonsils, adenoids, sinuses, teeth, and even the gastrointestinal canal, and transported into the general circulation, with consequent insults to circulatory tissues and articular surfaces, has been fascinatingly suggested by many investigators, albeit, hypothetically, in the absence of a specific and demonstrable toxic substance.

Our present knowledge of soluble toxins produced by certain streptococci, notably Streptococcus scarlatinae and Streptococcus erysipelatis, is narrowly circumscribed by the newness of this subject and the vast difference in the behavior of streptococcal soluble toxins and the better understood and more potent toxins produced by the diptheria and tetanus bacilli. Although groups of subtypes of Streptococcus scarlatinae have been demonstrated by means of agglutination and absorption of agglutinins, one is struck by the fact that a scarlet fever antitoxin, although produced with a single lot of scarlet fever toxin, is able to neutralize completely the toxins produced by serologically different strains of Streptococcus scarlatinae. Likewise it is universally agreed that the clinical picture in scarlet fever is a constant entity, although recent studies have demonstrated that the causative virus may be serologically differentiable from other true types of Streptococcus scarlatinae. The one indisputable criterion for the specificity of the Streptococcus scarlatinae is the toxin-antitoxin neutralization, rather than the serological tests for specificity, such as the agglutination and absorption of agglutinins. Practically all observers are convinced that the scarlet fever antitoxin, with proper antitoxic titer, is a specific neutralizing and curative serum, which when given early in the disease, preferably during the first three days of the illness, approaches the beneficient action of diphtheria antitoxin. The existence of subtypes of toxins produced by a group of scarlet fever streptococci has been demonstrated by Kirkbride and Wheeler.[8] From a series of observations on specific neutralization with scarlet fever serum, Park[9] has recently suggested the existence of groups of streptococci which produce nonidentical soluble toxins, although such streptococci were found to be antigenically related by agglutination reactions. Under such apparent and seemingly confusing notions about the specific role of Streptococcus scarlatinae in the production of scarlet fever, the hypothesis is advanced by the author, that a specific soluble toxin may possibly be produced by a group of serologically unrelated streptococci and that the clinical and pathological picture in rheumatic fever may be provoked by a prolonged fixation of this toxin in certain tissues. This conjecture should not be wholly foreign or unwarranted in the light of the new facts we have recently come to learn about the etiology of scarlet fever.

Swift and Kinsella[6] in their original article on rheumatic fever suggested such dual etiologic agents, namely, the streptococci on the one hand, and some unknown agent on the other hand.

The effect on the course of recurrent acute rheumatic arthritis of the thorough removal of the inflammatory processes in the upper air

[8] Proc. Soc. Exper. Biol. & Med., 1925, 22, p. 86; J. Immunol., 1926, 11, p. 477.
[9] J. Immunol., 1925, 10, p. 829.

passages, or hypothetically, the foci of toxin production has recently been followed by Holsti [10] during a period of four and a half years of constant observations of 35 treated cases of rheumatic fever. In this series no true relapses of acute rheumatic arthritis occurred in any purely rheumatic case which had reached puberty. The course of the rheumatic disease in adults was decidedly more benign after the removal of inflammatory processes in the upper air passages.

METHODS OF ISOLATION OF THE NON-METHEMOGLOBIN-FORMING STREPTOCOCCI

Following the isolation of strain RF. 1, an extensive study was undertaken to determine the frequency with which similar organisms could be isolated from patients with definite histories of acute rheumatic fever and its syndromes.

More than 400 strains of hemolytic and nonhemolytic streptococci were isolated from tonsillar crypts before and after tonsillectomy, from abscesses, urine, feces, blood, and heart vegetations. Excepting the blood cultures and heart vegetation cultures, the swabs with which cultures were made, were immediately placed in tubes containing 10 cc. of broth (P_H 7.6). By gently rolling the swab along the sides of the tube, an emulsion was made in the broth with the material absorbed by the swab. From this emulsion 0.5 cc. were removed and placed in a second tube containing 10 cc. of broth. After throughly mixing the contents in the second tube, 0.5 cc. of the mixture were removed and placed in a third tube containing 10 cc. of broth and mixed well. From each of the three tubes 0.1 cc. was removed into separate sterile 20 cc. test tubes, and to each of these was added about 15 cc. of molten 5% blood agar. After rapidly mixing the blood agar with the bacterial emulsion, the contents of the tube were poured into sterile Petri dishes and allowed to harden during half an hour, before they were placed in the incubator (37 C.). By this method one is always certain to obtain one plate with properly dispersed individual colonies. Suspected colonies of non-methemoglobin-forming gram-positive cocci are observed to best advantage in plates containing fewest colonies and such colonies are readily picked and streaked on other blood plates in pure culture. When blood was removed from patients for the purpose of culture, sterile surgical technic was always observed, and the blood was immediately poured into previously molten beef infusion agar, 3%, with P_H 7.8, and after mixing the blood with the agar, it was poured into Petri dishes and allowed to harden well before they were placed in the incubator at 37 C. Usually 5 cc. of the patient's blood were placed in 100 cc. of the agar, and the temperature of the molten agar was always reduced to about 50 C. before the addition of the blood took place. In three positive blood cultures the streptococcic colonies became visible in the blood agar plates within 24 hours and quite distinct in their reaction toward blood in 48 hours after incubation was initiated. The experience with blood cultures over a period of four years makes the author hesitant about growths in blood agar plates subsequent to 72 hours' incubation. This is in particular true of blood broth cultures, in which repeated opening of the flasks

[10] Acta Med. Scandinav., 1927, 65, p. 352.

for the purpose of fishing out medium for smears render the culture liable to contamination. The cultures obtained post mortem from vegetations on the mitral valves were made by carefully excising the desirable tissues under aseptic precautions and by immediately placing a part of the tissue in 100 cc. of broth. Another portion of the vegetation was placed in a tube containing 10 cc. of broth and with a rigid platinum spade the tissue was crushed against the sides of the tube until a turbulent emulsion had formed. Following the technic described under ordinary swab cultures, properly diluted portions of this emulsion were poured into 5% blood agar and placed in Petri dishes. The advantage of this method over that of placing the tissue directly into broth is that in the presence of contaminations, so commonly present at post mortem examinations, individual colonies are easily discerned and picked in pure culture. Cultures of rheumatic nodules were treated similarly to that of heart vegetations. No positive cultures were obtained from four excised nodules. In two instances, the excised nodules were buried subcutaneously in guinea-pigs. Daily study of the blood, feces and urine, both culturally and under the darkfield microscope, failed to reveal anything unusual during a period of eight weeks observation.

Ninety-eight strains of Streptococcus hemolyticus, or Streptococcus pyogenes (Schottmüller) were isolated from the tonsillar crypts of rheumatic fever patients and conformed in general with the morphological and cultural characteristics of this group of microorganisms. When grown in tryptic digest medium for more than five days, this group consistently failed to produce a soluble toxin demonstrable in the skin of individuals highly susceptible to the Dick toxin and the toxin produced by Streptococcus erysipelatis.

Among 315 strains of nonhemolytic streptococci isolated from rheumatic fever patients, 247 strains conformed with Streptococcus viridans type, in that they produced a zone of greenish discoloration and partial hemolysis in the blood agar plates after 48 hours of incubation at 37 C. The remaining 68 strains of nonhemolytic streptococci produced no apparent change in the blood until after eight to ten days of incubation, when a delicate semitransparent halo appeared around the central grayish-white colonies. No hemolysis nor definite discoloration of the blood agar was at any time observed with this particular type of microorganism. When human blood, or blood of guinea-pigs, sheep or dog was used instead of defibrinated rabbit blood, the organism remained inactive in such mediums without any methemoglobin-formation. Similar organisms were described by Mandelbaum [11] in 1907 and as Streptococcus saprophyticus; by Zangemeister [12] in 1910 as Streptococcus anhemolyticus vulgaris; by Rosenow [13] in 1914 in a series

[11] Ztschr. f. Hyg. u. Infektionskrankh., 1907, 58, p. 26.
[12] Die bakteriologische Untersuchung im Dienste der Diagnostik und Prognostik der Puerperalen Infectionen, 1910.
[13] J. Infect. Dis., 1914, 14, p. 61.

of organisms isolated from the joint fluid of cases with acute rheumatism, articular and muscular, and while my studies were in progress, by Small,[14] as Streptococcus cardioarthritidis.

Of the 247 strains of Streptococcus viridans grown for more than 5 days in tryptic digest medium for toxin production, only 11 strains (4.7%) produced positive skin reactions in dilutions of 1 to 100, when injected into the skin of susceptible persons. On the other hand, among the 68 strains of the non-methemoglobin-producing streptococcus, 49 strains (72%) gave strongly positive skin reactions in dilutions higher than 1 to 100, and at least 4 strains produced a soluble toxin reacting positively in 1 to 1,000 dilution in physiologic salt solution in susceptible persons. The lesions will be discussed under toxin production.

Agglutination Reactions of a Series of Non-Methemoglobin-Forming Streptococci with Strain RF. 1 Antiserum.—For the production of immune serum a combination of the methods described by Stevens and Dochez,[15] and Dochez [16] was employed. After a three months' course of immunization, the agglutinin titer of the serum against the RF. 1 strain employed was 1 to 5,120. The same methods were employed for the production of agglutinating serums against one strain of Streptococcus viridans (SV. 76) isolated from a case of subacute bacterial endocarditis, and another strain of Streptococcus viridans (R. 37) isolated from the blood of a case of acute rheumatic fever. The average agglutinin titer of the serum with the streptococci employed was 1 to 5,120. Since agglutination studies with large series of Streptococcus viridans isolated from rheumatic fever patients have failed consistently to reveal type specificity within groups of the methemoglobin-producing organisms, our chief concern was to study preliminarily the serological reactions of the non-methemoglobin-forming streptococci, isolated from the blood, tonsillar crypts, abscesses, urine and feces of rheumatic fever patients. Among the 68 strains of the non-methemoglobin-forming streptococci, 51 strains, or 75%, were agglutinated in various dilutions by the RF. 1 antiserum. Table 1 illustrates that the group of the non-methemoglobin-forming streptococci constitutes a culturally and serologically compact group, distinguishable by these methods from the Streptococcus viridans group.

The antiserums for the Streptococcus viridans type were produced with strains SV. 76, and R. 37, isolated respectively in blood cultures

[14] Small, J. C.: Am. J. M. Sc., 1927, 173, p. 101.
[15] J. Exper. Med., 1924, 40, p. 253.
[16] J. Am. M. A., 1924, 82, p. 542.

from cases of subacute bacterial endocarditis and acute rheumatic fever. Dr. Homer F. Swift, of the Rockefeller Institute for Medical Research, New York, kindly sent me strains A-49, B-38, 38-D, A-179, and W-72; the cultural and serological reactions of these cultures were fully described in his original article.[17] Strains B-38 resembled the non-

TABLE 1

BIOLOGICAL REACTIONS INCLUDING SUGAR-FERMENTATIONS, TOXIN PRODUCTION, TOXIN-ANTITOXIN NEUTRALIZATION, AND AGGLUTINATION REACTIONS OF NON-HEMOLYTIC STREPTOCOCCI ISOLATED FROM PATIENTS WITH HISTORY OF RHEUMATIC FEVER

Strain	Source	Glucose	Lactose	Sucrose	Mannitol	Salicin	Raffinose	Inulin	Bile	Toxin	RF.T-AT† Neutral	RF.1 Serum	Agglutination Reactions Streptococcus viridans Serum SV 76	R. 37
*Beattie-227	Blood	A	A	A	A	A	—	—	—	—	—	—†	—	—	—
*Allen-304	Blood	A	A	A	A	A	—	—	—	—	—	—	—	—	—
*Swift-A.49	Blood	A	A	A	—	—	—	—	—	—	—	—	—	—	1:40
*Swift-B.38	Blood	A	A	A	—	A	A	A	—	—	—	—	1:80	—	—
*Swift-38 D	Blood	A	A	A	—	—	—	—	—	—	—	—	—	—	—
*Swift-A.179	Heart	A	A	A	—	—	—	—	—	—	—	—	—	—	1:160
*Swift-W.72	Nodule	A	A	A	—	—	A	—	—	—	—	—	1:40	—	—
RF.1-b.	Blood	A	A	A	—	A	A	A	—	+	+	1:1,280	1:20	1:40
RF.1-v.	Heart	A	A	A	—	A	A	A	—	+	+	1:2,560	1:20	1:20
RF.2	Tonsil	A	A	A	—	A	A	A	—	+	+	1:640	—	—
RF.3-t.	Tonsil	A	A	A	—	A	—	A	—	+	±	1:160	—	—
RF.3-f.	Feces	A	A	A	—	A	A	A	—	+	+	1:320	1:20	1:40
RF.4	Tonsil	A	A	A	—	A	A	A	—	±	±	1:40	1:20	1:20
RF.5	Chorea	A	A	A	—	A	—	A	—	+	+	1:80	—	—
RF.6	Chorea	A	A	A	—	A	—	A	—	+	+	—	—	1:40
RF.7	Tonsil	A	A	A	—	A	—	A	—	—	..	—	1:40	—
RF.8	Tonsil	A	A	A	—	A	A	A	—	+	+	1:80	1:20	—
RF.9-t.	Tonsil	A	A	A	—	A	—	A	—	+	+	1:160	—	—
RF.9-f.	Feces	A	A	A	—	A	—	A	—	+	+	1:80	—	1:160
RF.10-b.	Blood	A	A	A	—	A	A	A	—	+	+	—	—	—
RF.10-t.	Tonsil	A	A	A	—	A	A	A	—	+	+	1:320	—	—
RF.10-a.	Abscess	A	A	A	—	A	—	A	—	+	+	1:1,280	1:20	1:40
*RF.11	Blood	A	A	A	—	—	—	—	—	—	—	—	—	—
RF.12	Tonsil	A	A	A	—	—	—	A	—	±	±	1:40	1:20	—
RF.13-a.	Abscess	A	A	A	—	A	—	A	—	+	±	1:640	1:40	1:80
RF.13-t.	Tonsil	A	A	A	—	A	—	A	—	+	+	1:320	1:40	—
RF.14	Tonsil	A	A	A	—	—	—	A	—	+	+	1:40	1:20	—
RF.15	Tonsil	A	A	A	—	A	A	A	—	+	+	—	—	—
RF.17	Chorea	A	A	A	—	A	—	A	—	+	+	1:640	1:40	1:20
RF.18	Abscess	A	A	A	—	A	A	A	—	+	+	1:1,280	1:40	—
RF.21	Tonsil	A	A	A	A	—	—	A	—	±	±	1:320	—	—
RF.24-t.	Tonsil	A	A	A	—	A	—	A	—	+	+	1:80	—	—
RF.24-u.	Urine	A	A	A	—	A	—	A	—	—	—	1:160	—	1:160
RF.36	Tonsil	A	A	A	—	A	A	A	—	+	+	1:1,280	1:80	—
*R.37	Blood	A	A	A	—	—	—	—	—	—	..	1:20	1:20	1:2,560
*SV.76	Blood (SBE)	A	A	A	—	—	—	—	—	—	—	1:20	1:2,560	1:40

* Typical strains of Streptococcus viridans type; remaining strains are non-methemoglobin-forming streptococci.
† T-AT neutral indicates that 0.1 cc. of the RF.1 antitoxin (rabbit) is mixed with 0.1 cc. of a 1 to 100 dilution of the toxic filtrate and is injected intradermally. + indicates complete neutralization; ± partial neutralization, and — no neutralization. A indicates acid fermentation without gas production. SBE implies subacute bacterial endocarditis.

methemoglobin-forming streptococci culturally and serologically, except in that it produced methemoglobin, and as such conformed to the type of Streptococcus viridans. With the exception of strains B-38 and W-72

[17] Arch. Int. Med., 1917, 19, p. 381.
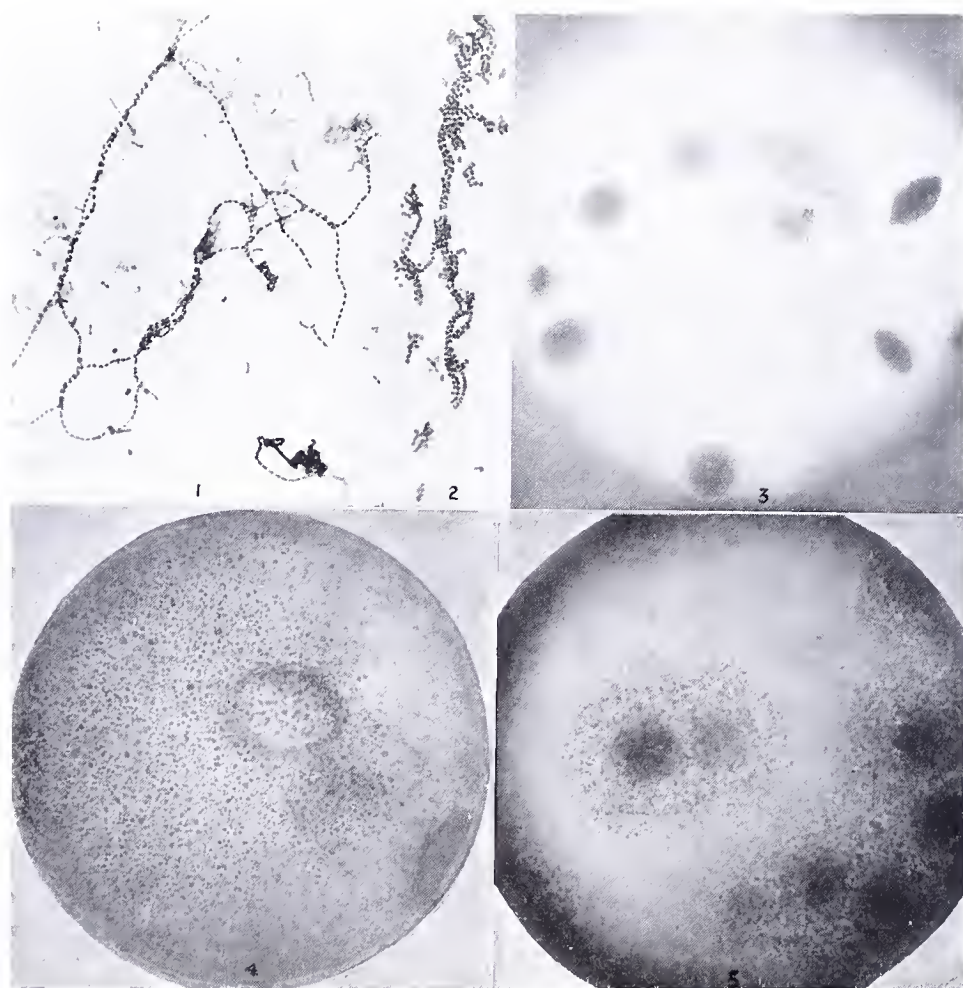
Fig. 1.—The non-methemoglobin-forming streptococcus RF. 1. × 1260.

Fig. 2.—The non-methemoglobin-forming streptococcus RF. 1, showing diplo-forms. × 1260.

Fig. 3.— Colonies of the non-methemoglobin-forming streptococcus RF. 1 in poured plates of dextrose-ascitic agar after 48 hours incubation. × 10.

Fig. 4.—Colony of the non-methemoglobin-forming streptococcus RF. 1 in poured blood agar plate, after 48 hours incubation, showing no hemolysis or discoloration. × 20.

Fig. 5.— Colonies of Streptococcus viridans in poured blood-agar plate, after 48 hours incubation, showing discoloration and partial hemolysis immediately surrounding the colonies. × 20.

which slightly agglutinated with the RF. 1 antiserum, a conclusive antigenic relationship of these strains with the RF. 1 type of non-methemoglobin-forming streptococci was not demonstrated by means of agglutination and absorption of the agglutinin. The Lister Institute, London, generously sent me their strains of Streptococcus rheumaticus, Allen-304, and Beattie-227. Poynton and Paine's Micrococcus rheumaticus, and Small's Streptococcus cardioarthritidis,[14] unfortunately, were not available for study and comparison. Culturally and serologically, the English strains of Streptococcus rheumaticus differed from the non-methemoglobin-forming streptococci.

The slight serological overlapping of the group of non-methemoglobin-forming streptococci with the Streptococcus viridans strains isolated from cases of subacute bacterial endocarditis and acute rheumatic fever, is too slight to indicate a convincing and definite antigenic relationship among these streptococci. One must await the results of more extensive immunological studies of these streptococci before definite conclusions about antigenic similarities and dissimilarities can be made.

Cultural Characteristics of the Non-Methomoglobin-Producing Streptococcus Isolated from Persons with History of Rheumatic Fever. —The inulin-fermenting and non-methemoglobin-producing organism isolated from a variety of sources in persons with definite history of rheumatic fever is a gram-positive coccus which normally forms short chains when grown in broth and occasionally appears in diplo forms.

The coccus is spherical or oval in shape, its diameter varying between 0.5 and 1 micron. It has no capsule, spore nor flagellum. In older cultures pleomorphic types frequently occur. The coccus is a facultative anaerobe. The most favorable temperature for growth lies between 30 C. and 37 C. On blood agar the culture survives in the ice-box at 2 C. about one month. When exposed to 60 C. for fifteen minutes the organism is killed. Gelatin is not liquefied and the growth of the coccus along the stab of the needle is delicately grayish-white and individual colonies are pinpoint in size. The organism does not grow on plain agar, and only sparingly in peptone water. Milk is coagulated. When planted in beef infusion broth, P_H 7.6, it grows diffusely during the first 24 hours at 37 C. After 48 hours' incubation a finely granular precipitate falls to the bottom of the flask. The supernatant fluid remains cloudy indefinitely. Addition to the beef infusion broth of glucose to 1%, greatly accelerates the growth of the organism, with rapid formation of acid. When left in the incubator for three to four days in glucose broth, death of the organism takes place. When streaked on blood agar plates containing 5% defibrinated rabbit blood in 3% beef infusion agar, growth is abundant within 24 hours. The surface colonies appear discrete, regularly outlined, their surfaces being moist, glistening and whitish-yellow in color. The center of the colonies is raised and the convexity is uniformly smooth and even. The size of individual colonies varies

between minute pinpoints and 1 mm. in diameter, resembling the surface growth of
Streptococcus hemolyticus except for the significant absence of hemolysis. The
colonies adhere readily to the platinum loop and when removed from the blood
agar surface, no change is observed in the underlying medium. When cultures
on blood agar plates are left in the ice-box over four days, a delicate, semi-
transparent and colorless halo surrounds the central spherical or oval colony.
The halo measures about 1 mm. in diameter about the eighth day in the ice-box.
The organism grows well on Loeffler's blood serum and most abundantly on
dextrose-ascitic-beef-infusion-agar, which contains about 10% ascitic fluid and
1% dextrose. The organism ferments dextrose, lactose, saccharose, salicin and
inulin regularly, and raffinose irregularly. It is bile insoluble. The organism
should be transferred weekly to dextrose-ascitic agar or to 5% blood agar.

*Inulin-Bile-Ascitic-Agar Medium for the Isolation of the Non-
Methemoglobin-Forming Streptococcus and Elimination of the Pneumo-
coccus.*—In order to facilitate the isolation in pure culture of the
non-methemoglobin-forming streptococcus from the blood agar plate and
to differentiate this organism at once from the pneumococcus, excellent
results have been obtained with an inulin-bile-ascitic-agar medium made
up as follows:

3% beef extract agar, Pн 7.8... 100 cc.	Ascitic fluid or beef serum....... 10 cc.
20% inulin, aqueous solution.... 5 cc.	Ox bile .... 5 cc.
Brom-cresol purple, saturated alcoholic solution	

To 100 cc. of melted agar, cooled to about 50 C., add 5 cc. of the 20% solu-
tion of inulin, 10 cc. of the ascitic fluid, or beef serum, and 5 cc. of the ox bile.
Mix thoroughly by agitating the flask on the waterbath at 50 C., and slowly add
to the mixture brom-cresol purple until the desired deep purple color is obtained.
This mixture is immediately poured into test tubes containing proper dilutions
of the bacterial emulsion made from throat swabs, abscesses, urine and feces.
It is necessary to add about 20 cc. of the medium to each tube in order to insure
an adequate depth when poured into Petri dishes. The poured plates are allowed
to harden during 15 to 30 minutes and are then placed in the incubator. Follow-
ing 24 hours' incubation, it is noted that the inulin-fermenting and bile-insol-
uble streptococci are conspicuously surrounded by a bright greenish-yellow halo,
which measures about 2 to 4 mm. in diameter. For pure culture purposes, such
colonies are readily picked.

If a differential study of the action of streptococci on blood agar is
desired, then the above described inulin-bile-ascitic-agar medium is first
poured into Petri dishes and allowed to harden in the ice-box before the
plates are employed for surface streakings of pure cultures. Suspicious
colonies of the non-methemoglobin-forming gram-positive cocci are then
picked from the blood agar plate and streaked across the surface of the
inulin-bile-ascitic-agar medium. Allow at least 2 cm. interspace between
each subculture because of the rapidly disseminating discoloration of the
inulin-fermenting organisms. The pneumococci fail to grow on this

medium and the non-methemoglobin-forming streptococcic colonies are conspicuous by the lemon yellow discoloration surrounding each individual colony. By eliminating the ox bile and substituting beef serum for the ascitic fluid, since the latter almost always contains traces of bile, an ideal medium is available for the study of the inulin-fermenting ability of the pneumococcus.

PATHOGENICITY OF THE NON-METHEMOGLOBIN-FORMING STREPTOCOCCUS

The virulence of the non-methemoglobin-forming streptococcus when injected intraperitoneally in mice is exceedingly low, 1 cc. of a 24 hours' broth culture killing the mice in 12 hours. When injected intravenously in rabbits, 15 to 20 cc. of a 24 hours' broth culture kills the animal in 24 to 48 hours. When the organisms are injected into rabbits by Dochez' method [16] of inoculating the living organisms into previously molten agar injected intramuscularly, it was found that 10 cc. of a 24 hours' broth culture mixed with 10 cc. of a 5% rabbit blood agar mass, killed rabbits regularly in the course of two weeks. If during the first week, after the intramuscular injection of the blood-agar-bacterial mass, a dose of 10 cc. of a 24 hours' broth culture was injected intravenously, fatal acute bacterial endocarditis, myocarditis, with massive vegetations on the mitral valves invariably occurred. The endocarditis eventually produced obstructive mitral stenosis, and systemic signs of myocardial insufficiency. The tricuspid valves were regularly left intact. Microscopic examination of the myocardium of rabbits succumbing to the inoculation with non-methemoglobin-forming streptococci isolated from rheumatic fever patients, revealed numerous small areas of cellular infiltration in the interstices of the muscle fibers. Many of these areas constituted small groups of large mononuclear cells, which formed characteristic arrangements of the nuclear material. About several of the smaller vessels were observed small areas of muscle degeneration, and in connection with these were seen collections of polymorphonuclear and mononuclear cells with tendency to clump into multinuclear forms. From the vegetations on the mitral valves, a pure culture of the non-methemoglobin-forming streptococcus was isolated, identical to that employed weeks previously in the intramuscular and intravenous injections of the animals.

The usual forms of inflammatory and nonsuppurative polyarthritis which follow close upon injections of massive doses of streptococci in
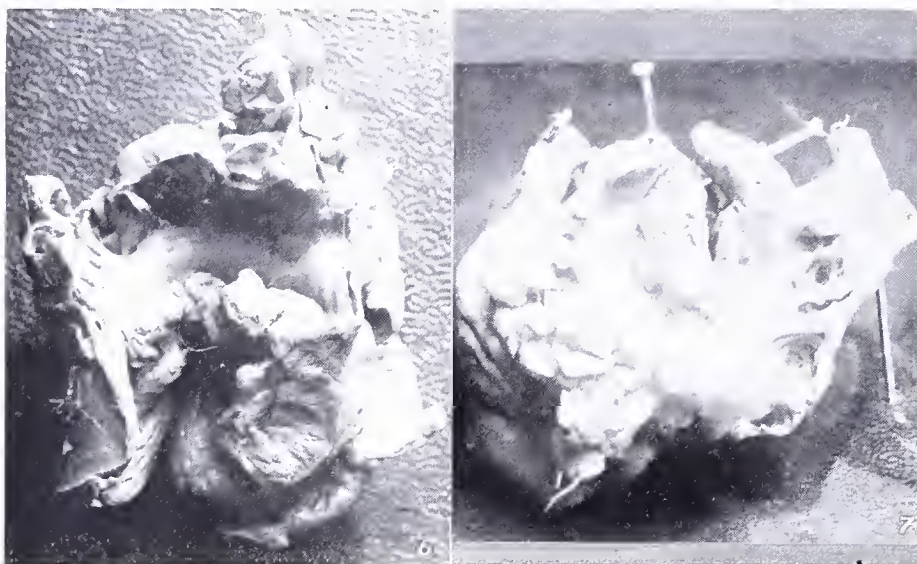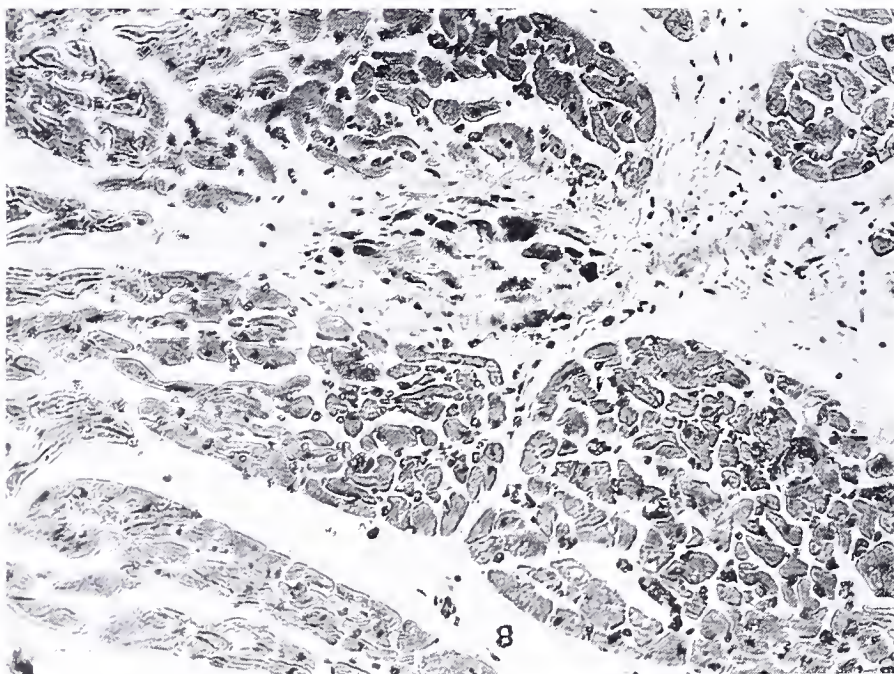
Fig. 6.—Rabbit 76-4. Endocarditis following intravenous injections of the non-methemoglobin-forming streptococcus RF. 1. Massive vegetations on auricular wall and mitral valve. × 2.

Fig. 7.—Same as figure 6, viewed from the auricular surface. × 2.

Fig. 8.—Rabbit 76-4. Myocardium showing focal collection of large mononuclear cells in the interstices of the muscle fibers. × 430.

rabbits, and described fully by Topley and Weir [18] were constantly observed. This streptococcus was never obtained from repeated cultures of the fluid from the affected joints. Swelling of the soft tissues usually took place about the shoulder and elbow joints, and occasionally all the larger joints became involved, to the extent that the animal became immobile or limped about slowly. Subsequent to the disappearance of the acute symptoms of the involved joints, no suppuration was encountered, except in such joints that became infected following repeated punctures for the purpose of obtaining cultures from the joint fluids. At necropsy the synovial membranes were found deeply injected and in the acute stages markedly thickened. Microscopically the synovial membranes showed infiltration of polymorphonuclear cells and in the older cases a marked proliferation of connectitve tissue cells. In only one instance were bony changes encountered, simulating acute osteo-chondritis. This animal was heavily inoculated over a period of four months and had been previously employed in studies with hemolytic streptococci.

Apropos of the experimental production of a pathological picture in the rabbit which simulates that obtainable in human cases of rheumatic fever, it must be borne in mind that the modus operandi differs decidedly in the rabbit from that functioning in human rheumatism. In humans stigmatized by rheumatic fever, one might suppose with Coombs,[19] "that a series, of varying length, of small doses (of the infective agent) escapes into the circulation through the tonsils, or some similar portal of entry." It becomes imperative then, if animal experimentation may possibly yield the pathological picture of typical rheumatic fever, to create conditions of infecting animals with whatever virus may be considered the etiologic agent, in a manner approaching that which operates in humans. It has, therefore, been suggested that rather than to make use of massive doses of the organisms, or their byproducts, such as soluble toxins, for the infection of the animal, the antigen might be buried within the animal body in a dialyzable sac, such as was described by Gates.[20] From such a membranous sac the living organism would constantly eliminate toxins, without the organism itself invading the body. Such studies are under consideration and may possibly be carried on in higher animals than the rabbit.

[18] J. Path. & Bact., 1921, 24, p. 333.
[19] Rheumatic Heart Disease, 1924, p. 16.
[20] J. Exper. Med., 1921, 33, p. 25.

TOXIN PRODUCTION OF THE NON-METHEMOGLOBIN-
FORMING STREPTOCOCCUS

A brief and adequately comprehensive review of the toxic products of streptococci was recently given by Zinsser.[21] The failure of early investigators to discover a streptococcic soluble toxic substance sufficiently potent to explain the symptoms of known streptococcic infections was largely due to the insusceptibility of laboratory animals to massive doses of streptococcic toxins. Recent studies of such soluble toxic substances directly upon man have profoundly altered our views regarding streptococcic toxins and their specific relationship to such diseases as scarlet fever, erysipelas, puerperal fever[22] and possibly measles.[23] In a valuable study of cutaneous reactions with soluble toxins produced by a large series of streptococci, Jacobsohn[24] observed that persons showing Dick positive reactions with Streptococcus scarlatinae toxin (1 to 1,000 dilution) failed consistently to react positively with 1 to 100 dilution of filtrates

TABLE 2

SKIN REACTIONS WITH FILTRATES FROM STREPTOCOCCUS CULTURES IN NORMAL PERSONS

Source of Filtrate	Dilution	Adults	Children	Positive	Negative	% Positive
Streptococcus scarlatinae.........	1:2000	148		42	106	28
			125	55	70	44
Streptococcus erysipelatis.........	1:1000	148		31	117	21
			135	20	105	16
Streptococcus viridans............	1:1000	113		..	113	0
			128	..	128	0
	1:100	110		7	103	6
			108	9	99	8
	1:10	54		6	48	11
			62	9	53	14
RF.1 (non-methemoglobin-forming streptococcus)	1:1000	118		11	107	9
			84	3	81	4
	1:100	118		18	100	15
			84	9	75	11
	1:10	65		12	53	18
			72	8	64	11

from hemolyic and non-hemolytic streptococci isolated from various infectious diseases other than scarlet fever. Recently, Kreidler[25] found that streptococci isolated from cases of endocarditis produced no exotoxin, endotoxin, or poisonous split proteins. The present study of the toxin production of the non-methemoglobin-producing streptococcus isolated from cases with rheumatic fever was carried on simultaneously with a comparative study of the toxin production of Streptococcus scarlatinae (Dick toxin), Streptococcus erysipelatis, and a number of Streptococcus viridans strains isolated from healthy and diseased individuals. The results of these studies are given in tables 2 and 3.

The toxins employed in these studies were prepared in Douglas' tryptic digest medium,[26] modified by Hartley,[27] Watson and Wallace[28] and extensively used for the production of diphtheria, scarlet fever and erysipelas toxins. The cultures were incubated at 37 C. for six days, when 0.3% phenol was added and

[21] Textbook of Bacteriology, 1927, chapt. 23.
[22] Lash, A. F., and Kaplan, B.: J. Am. M. A., 1925, 84, p. 1991.
[23] Tunnicliff, R., and Taylor, R. E.: J. Am. M. A., 1926, 87, p. 846; Ferry, N. S., and Fisher, L. W.: Ibid., 1926, 86, p. 932; Eagles, G. H.: Brit. J. Exper. Path., 1926, 7, p. 286.
[24] Acta Paediat., 1926, 6, p. 67.
[25] J. Infect. Dis., 1926, 39, p. 186.
[26] Lancet, 1914, 2, p. 891.
[27] J. Path. & Bact., 1922, 25, p. 479.
[28] Ibid., 1923, 26, p. 447.

the cultures were filtered through a Berkefeld V candle. The filtrate was then tested for sterility and stored away in the ice-box for one month before it was used experimentally on humans.

It was noted that the skin reactions with filtrates from Streptococcus scarlatinae and Streptococcus erysipelatis (hemolytic streptococci) as well as Streptococcus viridans were positive, in the same percentage, in normal persons and in persons with definite histories of rheumatic fever. On the other hand, when normal persons were tested with filtrates from the non-methemoglobin-producing streptococcus isolated from rheumatic fever patients, it was noted that among 118 adults without history of

TABLE 3

Skin Reactions with Filtrates from Streptococcus Cultures in Persons with History of Rheumatic Fever

| Source of Filtrate	Dilution	Adults	Children	Positive	Negative	% Positive
Streptococcus scarlatinae.........	1:2000	18		4	14	22
			54	26	28	48
Streptococcus erysipelas..........	1:1000	18		3	15	17
			54	10	44	18
Streptococcus viridans...........	1:1000	18		..	18	0
			54	..	54	0
	1:100	18		1	17	5
			54	4	50	7
	1:10	18		1	17	5
			54	6	48	11
RF.1 (non-methemoglobin-form- ing streptococcus)	1:1000	18		3	15	17
			54	9	45	17
	1:100	18		10	8	56
			54	41	13	76
	1:10	18		12	6	67
			54	46	8	85

rheumatic fever, 18 persons, or 15% gave positive reactions with 0.1 cc. of a 1 : 100 dilution filtrate, and among 84 children without any history of rheumatic fever, 9 persons or 11%, reacted positively. The same percentage was found when 0.1 cc. of a 1 : 10 dilution filtrate was injected intradermally. When persons with rheumatic fever and its subacute syndromes were tested intradermally with 0.1 cc. of the toxic filtrate, it was found that 17% of children and adults reacted positively with 1 : 1,000 dilution filtrate, and among 18 children tested with 0.1 cc. of a 1 : 100 dilution, 8 persons, or 56% gave positive reactions, and of 54 adults tested with the same dilution, 41 persons, or 76%, reacted positively. When the dilution was lowered to 1 to 10, it was found that among 18 children tested, 12 persons gave strongly positive reactions, or 67%, and among 54 adults, 46 persons, or 85% reacted positively with the toxic filtrate. When dilutions of 1 : 100 of the filtrate were injected, the skin lesions were similar to that observed in the Dick test, except in four instances when the lesions appeared like erythema nodosum and in

the latter instances, the lesions persisted painful and deeply injected for about one week. Injections of 1 to 10 dilutions of the toxin caused a reddening of the skin as soon as two hours after the intradermal injection and in the course of 24 hours the lesion was swollen, deeply injected and itching. Two days later, nodular elevations were palpable and if situated near the wrist or the elbow, movements of these joints became painful. The nodules remained visible and palpable for as long as two weeks. Futher study of the nodular forms of skin lesions produced by the intradermal injection of the toxic filtrate during the acute attack of rheumatic fever is in progress and biopsy material will be studied carefully.

Thermal inactivation of the toxic filtrates produced in Douglas' tryptic digest medium was first detected after boiling the filtrate for 1 hour. Incubation on the waterbath at 56 C. for more than 6 hours only slightly reduced the toxicity of the filtrate. Exposing the toxin to daylight and room temperature for three months failed to destroy the toxic principle. The active toxic substance was completely removed with the fraction precipitated by 6 volumes of absolute alcohol. The resultant white and flaky precipitate was readily redissolved in normal saline solution and in this manner could be concentrated and purified with great ease.

Toxin-Antitoxin Neutralization.—Three children admitted to the Strong Memorial Hospital during the acute attack of rheumatic fever were carefully studied serologically during their illness. On admission all three patients reacted strongly with the toxin produced by strain RF. 1. When filtrates were made from their autogenous strains of non-methemoglobin-producing streptococci isolated from their tonsils, abscesses, and feces, these patients again gave strongly positive reactions with 0.1 cc. of 1 : 100 dilution. Samples of the patients' blood serum failed to neutralize the toxic substance when mixed in equal proportions. During convalescence and complete subsidence of acute symptoms, the skin tests with the toxin (1 : 100 dilution) became negative in two of the three children, and simultaneously, their blood serum agglutinated with their autogenous non-methemoglobin-producing streptococci in dilutions of 1 : 160 and 1 : 360 respectively, and with the strain RF. 1 in 1 : 80 dilutions. At this time, complete neutralization of 0.1 cc. of 1 : 10 dilution of their autogenous non-methemoglobin-producing streptococci was obtained by mixing the toxic substance with 0.1 cc. of the patients' serum. Incomplete neutralization was obtained when 0.1 cc. of 1 : 10 dilution of toxin was mixed with 0.01 cc. of the patients' serum and 0.001 cc. failed completely to neutralize 0.1 cc. of 1 : 100

dilution of the toxin. The toxin produced by the strain RF. 1 isolated from the heart vegetations and the blood of a fatal case of rheumatic fever, was likewise completely neutralized in similar dilutions when mixed with these patients' serum.

Rabbits heavily inoculated both intravenously and subcutaneously with the toxic filtrates of individual strains of non-methemoglobin-producing streptococci isolated from cases of rheumatic fever, rapidly produced a highly antitoxic serum. During the course of three months' immunization, four out of seven rabbits given injections of the toxic filtrate alone, developed nonsuppurative generalized arthritis. The

Chart: Clinical course following injection of toxic filtrate from non-methemoglobin-forming streptococcus strain RF. 1.
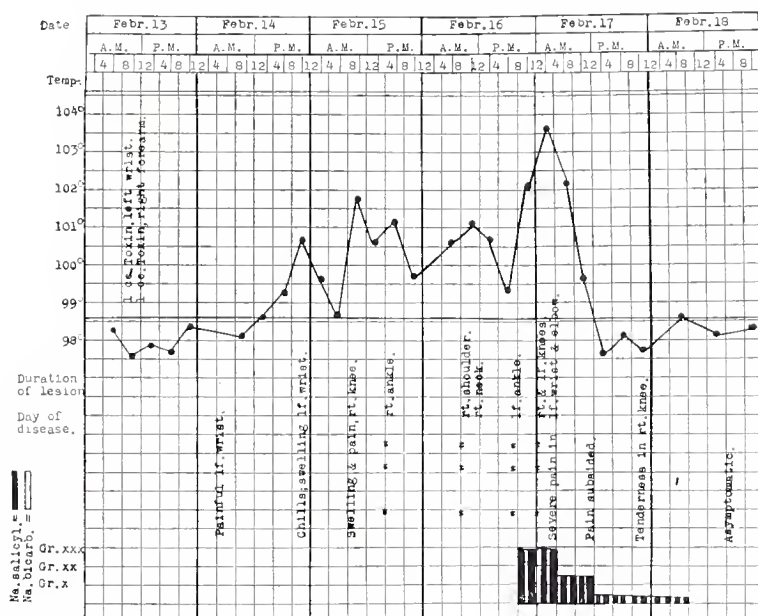
arthritic condition grew steadily worse until the animals were exsanguinated. No heart lesions were observed at necropsy, comparable to those commonly found in rabbits inoculated intravenously and subcutaneously with the living non-methemoglobin-producing streptococcus. The antitoxin produced with individual lots of toxic filtrates in rabbits, completely neutralized the toxins produced by other serologically and culturally related streptococci. Numerous controls with normal rabbits serum or immune rabbit serum heated at 56 C. for one hour, failed to neutralize individual lots of the toxic substance.

Two persons recently recovered from acute attacks of rheumatic fever and whose skin remained persistently hypersensitive to the toxic fil-

trate produced by strain R.F. 1, were injected at bi-weekly intervals subcutaneously with 0.01 cc., 0.1 cc., 0.5 cc., 1 cc., 5 cc. and 10 cc. of the R.F. 1 toxic filtrate, mixed with 0.1 cc. of convalescent rheumatic fever serum. A mild systemic reaction followed in both instances after injection of the 10 cc. dose of toxin, with swelling and tenderness in the right elbow and wrist in one person, and the right knee and left wrist in the other person. The swelling and pain disappeared during the next twenty-four hours. Ten days later both persons gave negative skin reactions with 0.1 cc. of a 1 : 10 dilution of RF. 1 toxic filtrate. The blood taken from these persons at this time, yielded a serum, 0.01 cc. of which completely neutralized 0.1 cc. of a 1 : 10 dilution of the RF. 1 toxic filtrate. During the following four months these patients were retested intradermally with the RF. 1 toxin and negative results were obtained in both cases. The influence of the toxin-antitoxin immunization on the course of pre-existing syndromes of rheumatic fever is not within the scope of this paper and such evaluation necessarily must await long periods of observation.

EXPERIMENTAL PRODUCTION OF ACUTE POLYARTHRITIS WITH THE SOLUBLE TOXIN PRODUCED BY THE NON-METHEMOGLOBIN-FORMING STREPTOCOCCUS

The Schick test in diphtheria, the Dick test in scarlet fever and a similar test demonstrated by Birkhaug[29] in erysipelas, are generally considered to indicate the existence of susceptibility to an attack of diphtheria, scarlet fever, or erysipelas, when such tests give positive reactions. Since August 1926, the author has regularly given a positive skin reaction with 0.1 cc. of a 1 to 100 dilution of the RF. 1 soluble toxin. No general reaction has ever followed the intradermal injections of these minute skin test doses of toxin. In order to satisfy his own curiosity about whether or not a positive skin reaction with the toxic filtrate affords an index of susceptibility to the syndromes encountered in an attack of acute rheumatic fever, with particular reference to the acute polyarthritic phenomenon, the author, on February 13, 1927, at 9 a. m., injected 1 cc. of sterile and purified soluble RF. 1 toxin directly into his left wrist joint. A similar dose was simultaneously injected intramuscularly in the right forearm.

Results: Except for an almost immediate reddening of the skin surfaces overlying the injected areas, no general symptoms developed during the day.

The following day, February 14, at 8 a. m., about 23 hours after the injections of the toxic filtrate, a feeling of sudden general malaise occurred, shortly followed by profuse sweating and marked dryness of the throat. The left wrist became throbbing and intermittently painful during the forenoon and at 11 p. m.,

29 Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 201.

chills developed without any rigor, the temperature rose to 100.6 F., and the pulse to 110. At this time the left wrist was definitely swollen and continuously painful; the right forearm was deeply injected about the site of the inoculation, the redness measuring about 10 cm. in diameter. Due to excessive swelling, the movement of the muscles were painful.

On February 15, at 8 a. m., following a drenching sweat during the early part of the morning, the temperature had fallen to 98.7 F. and the pulse to 95. The swelling and pain in the left wrist and right forearm persisted unabated. At 10 a. m., frontal headache and dizziness began, and gradually the right knee became tender, painful and swollen. The temperature rose to 101.8 F. and pulse to 118, and a very acid perspiration continued profusely. About 4 p. m., the right ankle became involved and sharp, stinging pains preceded the rapidly reddened overlying swollen tissues. Following a heavy perspiration, the temperature fell to 99.6 F. at midnight and the pain in the larger joints almost completely subsided.

On February 16, the feeling of general heaviness persisted. The pain and swelling in the left wrist and right knee had almost completely cleared up. At 12 o'clock noon, the temperature rose to 101 F., and the pulse to 118, while the pain suddenly shifted to the right shoulder and muscles in the neck. About 8 o'clock, p. m., the left ankle became exceedingly painful and swollen, and a depressive feeling of general malaise, complicated with throbbing frontal headache, dryness of the throat, thirst and profuse sour sweating, made the author default his plan to abstain from the use of salicylates until the arthritis was fully developed. The temperature at 10 p. m., had risen to 102.1 F., and the pulse to 124. Sodium salicylate, gr. xxx (2 Gm.) and sodium bicarbonate, gr. xxx (2 Gm.) were taken every hour during the following eight hours.

On February 17, at 2:30 a. m., the temperature was 103.6 F., and the pulse, 136. The pain became stinging in the right and left knees, and the left wrist and elbow. At 6 a. m., tinnitus and diarrhea developed. The dosages of sodium salicylate and sodium bicarbonate were reduced to gr. xv (1 Gm.) of each, and local application of oil of wintergreen to the left wrist and right knee were resorted to because of the intense pain in these joints. After a drenching sweat about 10 a. m., the temperature fell rapidly to 99.6 F., and the pulse to 84. Shortly afterward the pain and swelling almost disappeared. At 2 p. m., the temperature and pulse were normal, and except for slight tenderness in the left wrist and the right knee, the general condition was fairly satisfactory. Salicylate of soda (gr. v), every two hours, was continued until noon the following day, when the pain and swelling of the larger joints were mostly cleared up. A residual weakness in the left wrist and right knee persisted during the next five days, but without continuous pain.

The x-ray report on February 18, 1927, reads as follows: "Left wrist and right knee: There is no evidence of recent injury to the articular surfaces. There is marked increase in density in the soft tissues immediately around the joints, which may be due to recent inflammation."

A SECONDARY RECRUDESCENCE OF SWELLING IN THE SKIN PRE-
VIOUSLY INOCULATED WITH THE TOXIN PRODUCED BY THE
NON-METHEMOGLOBIN-FORMING STREPTOCOCCUS
ASSOCIATED WITH RHEUMATIC FEVER

During the incipient stages of arthritic involvement when the pain and swelling suddenly migrated from one joint to another, a curious phenomenon was observed in the areas of the skin inoculated a few days

previously with 0.1 cc. of a 1 : 10 dilution of the RF. 1 toxic filtrate. Initially, an itching sensation occurred in these areas, elsewhere described as resembling erythema nodosum, because of their slightly elevated and nodular appearance. Gradually, a deep red hue made these localized areas prominently conspicuous, and the elevation above the skin surface made them plainly visible at some distance during the height of the arthritic swelling and pain. As soon as the arthralgic attack subsided, the swelling and redness in the nodular skin lesions were reduced to the point of obscurity, although, the nodules remained slightly palpable. By carefully watching the reaction in these areas, the author soon learned that recrudescence of arthralgic activity was impending in the joints previously affected, or in new locations.

This reaction is strikingly analogous to the Arthus phenomenon,[30] or the "secondary reaction" described by Andrewes, Derick, and Swift,[31] and subsequently studied determinatively by Derick and Andrewes.[32] In the former phenomenon, Arthus observed that rabbits immunized against horse serum showed an acute localized inflammatory reaction in response to a subcutaneous injection of horse serum, which failed to produce any such reaction when injected into normal rabbits. In the later reaction, Andrewes, Derick and Swift observed that rabbits inoculated intradermally with certain strains of green streptococci have well marked lesions which, after reaching a maximum size in 24 to 48 hours and then beginning to retrogress, show in over 50% of the animals a secondary increase in size and other signs of inflammation about 8 or 9 days after inoculation. Inoculation with hemolytic streptococci failed to bring about this "secondary reaction" in the hands of these investigators. Whether the production of experimental acute polyarthritis, reported in this paper, and the phenomenon of secondary recrudescence of activity in skin areas previously injected with sterile toxic filtrates are one and the same phenomenon and possibly a form of allergy in relation to the group of non-methemoglobin-forming streptococci associated with rheumatic fever, is a matter for future study.

SUMMARY

A new species of an non-methemoglobin-forming, inulin-fermenting, bile-insoluble, and toxigenic, gram-positive streptococcus, was regularly isolated from the tonsillar crypts, abscesses, and irregularly from blood cultures, heart vegetations, feces and urine, of persons stigmatized by rheumatic fever and its syndromes.

[30] Compt. rend. Soc. de biol., 1903, 55, p. 817.
[31] J. Exper. Med., 1926, 44, p. 35.
[32] Ibid., p. 55.

Culturally, toxigenically and serologically, the non-methemoglobin-forming streptococci constitute a closely related group of micro-organisms, distinguishable biologically from the groups of Streptococcus viridans and Streptococcus hemolyticus.

In a study of the production of soluble toxins by streptococci isolated from rheumatic fever patients, it was found that among 98 strains of Streptococcus hemolyticus, no toxin was demonstrated, among 247 strains of Streptococcus viridans, 4.7% were toxin producers, and among 68 strains of the non-methemoglobin-forming streptococcus, 72% were found to produce a soluble toxic filtrate, slightly weaker in potency than that produced by the Streptococcus scarlatinae and Streptococcus erysipelatis.

Normal persons without history of rheumatic fever when injected intradermally with 0.1 cc. of a 1 to 10 dilution of the toxic filtrate of the non-methemoglobin-forming streptococcus, 18% of adults and 11% of children gave positive skin reactions measuring more than 1 cm. in diameter within 24 hours after the injection.

Among persons with definite history of rheumatic fever and its syndromes, when tested intradermally with 0.1 cc. of a 1 : 100 dilution of the soluble toxic filtrate produced by the non-methemoglobin-forming streptococcus, 56% of adults and 76% of children gave positive skin reactions, and when tested with 1 : 10 dilution of the toxin, 67% of adults and 85% of children reacted positively with lesions measuring more than 1 cm. in diameter within 24 hours after the injection.

Intramuscular injections of increasing doses of the toxic filtrate in laboratory animals and in humans susceptible to the toxin produced a neutralizing antitoxin.

Injected intravenously and subcutaneously in rabbits, the non-methemoglobin-forming streptococcus invariably produced nonsuppurative polyarthritis, subacute bacterial endocarditis, myocarditis and epicarditis, with unique tendency to mitral stenosis, extensive mural vegetations, and occasionally myocardial degeneration, with nodular cellular arrangements of multinuclear forms, as well as polymorphonuclear and mononuclear cells.

Injection intra-articularly and intramuscularly of large doses of the soluble toxic filtrate of the non-methemoglobin-forming streptococcus in the author who previously had given repeatedly a strongly positive intradermal reaction with 0.1 cc. of a 1 : 100 dilution of the toxin, produced a typical clinical picture of acute polyarthritis of the rheumatic type, which healed without suppuration or injury to the articular surfaces.

CLASSIFICATION OF BACTERIUM COLI

BASED ON THE STUDY OF 75 VARIETIES

Bruno Leo Monias

From Grant Hospital, Chicago

Considering the importance of the bacteria in the human intestines, an exact classification is desirable. In this first article a new classification of bacteria usually grouped under Bacterium coli is presented.

Rodet and Orlowski[1] found eleven transitional types between Bacterium coli and Bacterium typhosum. Gilbert[1] made classes according to motility, lactose fermentation and indol production. Lembke[1] made four groups (table 1).

TABLE 1

Four Groups of Bacterium Coli (Lembke)

	Motility	Sugar Fermentation	Indol Production	Pathogenicity
Bacterium coli commune..............	+	+	+	0
Bacterium coli anindolicum...........	+	+	0	0
Bacterium coli anaerogenes...........	+	0	+	+
Bacterium typhi abdominalis.........	+	0	0	+

TABLE 2

Classification According to Escherich

	Gas Production from Dextrose	Action on Lactose	Milk Coagulation	Indol Production	Flagella	Capsules
Bacterium faecale alcaligenes..	0	Alkaline	0	0	Many	0
Bacterium typhi abdominalis..	0	Weakly alkaline	0	0	Numerous	0
Bacterium coli a.......	+	Strongly acid	(+)	+	Several	0
Bacterium coli b..............	+	Strongly acid	0	+	Few	0
Bacterium coli immobile.......	+	Strongly acid	+	+	0	0
Bacterium lactis aerogenes.....	+	Very acid	+	0	0	+
Bacterium pneumoniae.........	+	Strongly acid	0	0	0	+
Bacterium scleromatis.........	0	0 or weakly acid	0	0	0	+
Bacterium lactis innocuum.....	0	Alkaline	0	0	0	+

Refik,[1] Ehrenfest,[1] Wilde,[1] and Escherich[1] classified a larger group of bacteria according to motility, milk coagulation, indol production and fermentation (table 2).

Jackson[2] used a large number of carbohydrates and made four types according to their ability to ferment sugars and to split dulcitol: bacterium communior, commune, aerogenes and acidi-lactici. He distinguished subfamilies according to motility and ability to split manitol and raffinose. Castellani[3] and Castellani and Chalmers,[4] using Jackson's method, studied families of this group but discarded Jackson's nomenclature for: Tribus ebertheae with genera as follows: alkaligenes, eberthus, shigella, etc.

[1] Escherich and Pfaundler: Kolle u. Wassermann, Handbuch der pathog. Mikroorganismen, 1903, 2, p. 334.
[2] J. Infect. Dis., 1911, 8, p. 241.
[3] Centralbl. f. Bakteriol., I, O., 1912, 65, p. 262.
[4] Ann. de l'Inst. Pasteur, 1920, 34, p. 600.

The following study of the morphology and the biology of 75 cultures obtained from the microbiologic collection of E. Pribram serves as the basis for a classification which permits the indentification of any culture belonging to this group.

Morphology.—Gram stain was used, the hanging drop and Zettnow's method to stain flagella. According to Zettnow[5] a brownish black precipitate of silver oxide is obtained upon the addition of a few drops of a 33% ethylamine solution diluted with an equal amount of water to a silver sulphate solution. The precipitate is redissolved by adding a small quantity of ethylamine. Using an old solution, the precipitate can be dissolved by adding a small quantity of best ethylamine or with the aid of a few drops of 3% ammonium hydroxide.

Biochemistry.—Examination for acidity and coagulation of milk was made during from 3 to 8 days. Indol was tested for by Ehrlich's method. To 5 cc. of inoculated broth, incubated for 8 days, are added 2.5 cc. of solution A* and 2.5 cc. of solution B.** A bright red color appears in a few. minutes, when indol is present. In addition the creatinine test was used to determine proteolytic and peptolytic changes. I used for this purpose a sterile solution of 2% peptone and a 0.5% sodium chloride;[6] the inoculated tubes, containing 10 cc. of this solution, were kept for 20 days in moist chambers (dessicators with moistened cotton) at a temperature of 37 C. Then to 5 cc. of the culture 4 drops of a freshly prepared 1% sodium nitroprusside solution and the same amount of sodium hydroxide were added. When creatinine is present the mixture turns a ruby red color, and upon the addition of a few drops of concentrated acetic acid it turns a Prussian blue.[7] Test for acid formation and gas production from carbohydrates was according to Halle and Pribram's[8] modification of Lindner's[9] method:

Sterilized strips of filter paper; hollow ground slides; an indicator consisting of 100 cc. sterile broth and 8 cc. of sterile 1% aqueous solution of Congo red; a mixture of 60% vaseline and 40% paraffin; a fine camel's hair brush in a sterile well corked test tube; a sterile 30 cc. pipet, each end of which is connected with a capillary glass by a rubber tube, the lower tube controlled by a pinchcock, with about 30 cc. of the sterile indicator drawn into the pipet, the rubber tube with the capillary glass, attached to the lower end and sealed over a low flame, the tip of the sealed ends broken off before using the indicator and sterilized over gas burner; sterilized watch glasses to cover the slides before using them; sterilized cover glasses in alcohol; carbohydrates: arabinose, dextrose, levulose, mannose, galactose, saccharose, maltose, lactose, raffinose, dextrin; and the alcohols, sorbitol, mannitol and dulcitol. The carbohydrates were added to the medium with a sterilized platinum loop, as experience has taught me that sugars taken from well sealed Kahlbaum or Merck bottles needed no further sterilization. A few drops of the inoculated indicator were placed into the cavity of the slide. The carbohydrates were then added and the slides covered with the cover glass, using the paraffin vaseline mixture to get air tight closure. After 6 to 8 hours at 37 C., acid formation can be recognized by the formation of a black precipitate and gas formation sometimes is so distinct that the entire field may be filled by one bubble. The sterility of the indicator and the carbohydrate is tested by a control preparation that is not inoculated.

Seventy-five cultures of different sources in Pribram's collection[10] were used. The original names appear in table 3.

According to their predominating properties cultures 1 to 44 form one main group, characterized by the complete fermentation (gas production)

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* Stock Solution A: p-dimethyl-amidobenzaldehyde .... 4 gm.
    Alcohol (96%) .... 380 cc.
    Hydrochloric acid (conc.).................................. 80 cc.
** Stock solution B: saturated aqueous solution of potassium bisulphate.

[5] Ztschr. f. Hyg. u. Infektionskr., 1899, 30, p. 595.
[6] German: Centralbl. f. Bakt., I, O., 1912, 63, p. 545.
[7] Salkowski: Ztschr. f. physiol. Chemie, 1886, 10, p. 399.
[8] Wien. klin. Wchnschr., 1916, 24.
[9] Mikroskopische Betriebskontrolle in den Gaehrungsgewerben, 1901.
[10] Literature on cultures used in this study. Pribram: Der gegenwaertige Stand der Kral'schen Sammlung, 1909.

TABLE 3
Original Names and Sources of 75 Cultures Used in Study

1.	Bacterium communior A	Winslow, New York
2.	Bacterium coli	Pollak, Vienna
3.	Bacterium communior rubrum	Winslow, New York
4.	Bacterium coli A3	Woll, Stockholm
5.	Bacterium coli beta polare	Lehmann, Wuerzburg
6.	Bacterium aerogenes	Maschek, Prague
7.	Bacterium coli A	Schnuerer, Vienna
8.	Bacterium aerogenes	Kornauth, Vienna
9.	Bacterium coli, from Emmenthal cheese	Lehmann, Wuerzburg
10.	Bacterium coli B15	Woll, Stockholm
11.	Bacterium 361	Viennese collection
12.	Bacterium coli B7	Woll, Stockholm
13.	Bacterium coli-flexner	Wagner, Munich
14.	Bacterium coli of urine	Sanatorium F., Vienna
15.	Bacterium coli 3	Kulka, Lublin
16.	Bacterium coli 13	Kulka, Lublin
17.	Bacterium coli A4	Woll, Stockholm
18.	Bacterium coli A2	Woll, Stockholm
19.	Bacterium agar	Kral, Prague
20.	Bacterium coli kidney	Soeldner, Vienna
21.	Bacterium coli A16	Woll, Stockholm
22.	Bacterium coli B8	Woll, Stockholm
23.	Bacterium atypical	Jaffee, Vienna
24.	Bacterium coli septicaemiae gallinarum	Zeiss, Giessen
25.	Bacterium communior luteum	Winslow, New York
26.	Bacterium coli mobile	Loewy, Bozen
27.	Bacterium cloacae	Jordan, Massachusetts
28.	Bacterium coli B14	Woll, Stockholm
29.	Bacterium coli haemolyticum 16	Kraus, Vienna
30.	Bacterium coli	Miessner, Hanover
31.	Bacterium coli from urine	Porges, Russia
32.	Bacterium coli haemolyticum II	Kraus, Vienna
33.	Bacterium coli kidney	Eigenfeldt, Vienna
34.	Bacterium coli	Schnuerer, Vienna
35.	Bacterium coli verum	Smyth, Philadelphia
36.	Bacterium coli 3	Kulka, Lublin
37.	Bacterium coli simile	Kulka, Lublin
38.	Bacterium acidi lactici A2	Winslow, New York
39.	Bacterium acidi lactici B	Winslow, New York
40.	Bacterium acidi lactici B2	Winslow, New York
41.	Bacterium acidi lactici Hueppe	Miessner, Hannover
42.	Bacterium coli B6	Woll, Stockholm
43.	Bacterium coli	Lederer, Vienna
44.	Bacterium coli Kind	Kraus, Vienna
45.	Bacterium coli from dough	Lehmann, Wuerzburg
46.	Bacterium coli histidin splitter	Twort, London
47.	Bacterium coli mobile	Loewy, Bozen
48.	Bacterium coli simile from urine	Rotky, Prague
49.	Bacterium coli mutabile	Neisser, Frankfurt
50.	Bacterium pseudocoli	Woll, Stockholm
51.	Bacterium lactis aerogenes	Escherich, Vienna
52.	Bacterium coli phenologenes	Rhein, Posen
53.	Bacterium coli 103	Arzt, Cracovie
54.	Bacterium coli 106	Arzt, Cracovie
55.	Bacterium coli, Camembert cheese	Lehmann, Wuerzburg
56.	Bacterium coli anaerogenes	Winslow, New York
57.	Bacterium coscoroba	Winslow, New York
58.	Bacterium candiense	Castellani, Colombo
59.	Bacterium madapense	Castellani, Colombo
60.	Bacterium lunavense	Castellani, Colombo
61.	Bacterium levans 2	Lehmann, Wuerzburg
62.	Bacterium levans Wolffin	Lehmann, Wuerzburg
63.	Bacterium talavense	Castellani, Colombo
64.	Bacterium coli simile I	Graef, Kiel
65.	Bacterium coli simile 346	Viennese collection
66.	Bacterium colombense Garvin	Castellani, Colombo
67.	Bacterium pseudoasiaticum	Castellani, Colombo
68.	Bacterium asiaticum 1	Castellani, Colombo
69.	Bacterium giumai	Castellani, Colombo
70.	Bacterium tangalense	Castellani, Colombo
71.	Bacterium paratyphosum Brzak	Jaffee, Vienna
72.	Bacterium icteroides Sanarelli	Winslow, New York
73.	Bacterium colombense Wild	Castellani, Colombo
74.	Bacterium levans Wolffin	Lehmann, Wuerzburg
75.	Bacterium lactis aerogenes	Jaiser, Stuttgart

of dextrose and lactose and the production of indol and creatinin. Four subdivisions were found in this group: (A) 1 to 8, which ferment all carbohydrates, corresponding to the group: Bacterium communior Jackson; (B) 9 to 13, which do not ferment saccharose and raffinose: Bacterium commune Jackson; (C) 14 to 27 which ferment all carbohydrates except dulcitol: Bacterium aerogenes Jackson; (D) 28 to 44 which do not ferment saccharose, raffinose and dulcitol: Bacterium acidi lactici Jackson.

A second main group, comprised of cultures 45 to 54, characterized by deficiency of indol and creatinin production. This group corresponding somewhat to Lembke's Bacterium anindolicum, may be called Bacterium coli, var. anindolicum. The other characteristics of lactose fermentation and milk coagulation of the group Bacterium coli are present. The subdivisions A, B, C, D of the first group are exemplified also in his group. There are cultures that ferment all sugars, those that do not ferment saccharose and raffinose, others that do not ferment dulcitol and finally cultures with a deficiency of ferments for saccharose, raffinose and dulcitol. In this group there is the tendency not to ferment levulose and in four cultures even the fermentation of dextrose is absent.

A third group, cultures 55 to 63, is well characterized by the inability to produce gas from any carbohydrate. The name, Bacterium anaerogenes, given by Lembke, may be used for this subdivision. This group forms a transition between the group of Bacterium coli and Bacterium enteritidis. Some representatives of this group coagulate milk, produce indol and creatinin, others do not. There is no gas formation and mostly no acid, or only a very weak acid production from lactose. The characteristics of the coli group therefore may be completely absent in some of these cultures.

In the cultures 64 to 75 the characteristics of the coli group, i. e., milk coagulation and lactose fermentation, even acid production, are absent. This group may be designated Bacterium enteritidis. Its positive characteristic are motility, peritrichous flagella, and inability to retain the Gram stain. There are two subdivisions: the representatives 64 to 70 form indol and creatinin, and this subgroup therefore is more related to the coli group, and the other group, 71 to 75, without these characteristics, is closely related to the group of Bacterium typhosum. The subdivisions A, B, C, D of the first and second main groups Bacterium coli and Bacterium anindolicum according to their ability to ferment or not saccharose, raffinose and dulcitol, will be found in the group of Bacterium enteritidis as well.

A graphic tabulation may facilitate the survey of the different groups of the motile gram-negative bacteria.

TABLE 4

CLASSIFICATION OF BACTERIA WITH COMMON CHARACTERISTICS: GRAM-NEGATIVE, MOTILE, WITH PERITRICHOUS FLAGELLA

	Bact. Coli		Bact. Anaerogenes Subgroups				Bact. Enteritidis
			1	2	3	4	
Milk coagulation...............	+		+	+	0	0	0
Lactose fermentation.........	+ (complete)		0	0	0	0	0
	1. Bact. indolicum	2 Bact. anindolicum	No carbohydrate fermentation				Subgroups
							1 2
Indol production...............	+	0	+	0	+	0	+ 0
Creatinin production..........	+	0	+	+	0	0	+ 0
	Varieties						Varieties
	A B C D A B C D						A B C D A B C D

A. Communior: gas production from all carbohydrates.
B. Commune: gas production from all carbohydrates, except saccharose and raffinose.
C. Aerogenes: gas production from all carbohydrates except dulcitol.
D. Acidi-lactici: gas production from all carbohydrates except saccharose, raffinose, and dulcitol.

SUMMARY

The first group, Bacterium coli, is characterized as follows: gram-negative, motile, peritrichous flagella, coagulation of milk and fermentation of lactose.

The examination of 75 strains gives two subdivisions: (1) strains that produce indol and creatinin (Bacterium indolicum) and (2) strains without these qualities (Bacterium anindolicum). Further subdivisions in both of these groups may be made according to their ability to ferment carbohydrates: varieties communior, commune, aerogenes and acidi lactici corresponding to Jackson's nomenclature.

A second group, Bacterium anaerogenes, is characterized by the same qualities as the group, Bacterium coli, but differs in inability to produce gas from carbohydrates. There are four subdivisions in this group, according to ability to produce indol and creatinin and to coagulate milk or partial or total inability to do so. This forms a transition group between Bacterium coli and Bacterium enteritidis.

The third group, Bacterium enteritidis, differs from Bacterium coli by the lack of power to coagulate milk and to ferment lactose. In this group as in the group, Bacterium coli, two subdivisions are found: one with indol and creatinin production, the other without. In each of these groups there are again four classes, A, B, C, D, according to the fermentation of saccharose, raffinose and dulcitol.

SEWAGE FILTRATE AS A SOURCE OF BACTERIOPHAGE

JANET ANDERSON CALDWELL

From the Baylor Hospital Laboratory, Dallas, Texas

In many studies of the bacteriophage, much time and energy has been spent obtaining suitable lytic principle. The adaption of a bacteriophage strain to a nonsusceptible organism is often a tedious and unsuccessful procedure. There is, in addition, the feeling that adapted bacteriophage is inferior to one which is active when isolated. Judging from recent articles on bacteriophagy, the isolation of active native bacteriophage has been one of the chief problems with which to contend, so that adaption, despite its drawbacks, has been the method of choice. The dysentery bacteriophage is one of the well known exceptions.

Despite the difficulty of isolation, active bacteriophage seems ubiquitous. It occurs in the gastrointestinal tract of man and many animals, in soil and surface waters, in the lesions and exudates of many diseases and is even carried in stock cultures through many transplants. Because these lytic principles are often of low virulence; or are specific, so that their action is limited to only one or two species or strains of bacteria, many sources may be searched before a virulent strain can be found for the organism to be studied.

In addition to these difficulties, certain erroneous conclusions are being introduced into the literature of the bacteriophage concerning the frequency and nature of resistent strains of a given bacterial species. If absence of lysis is to be considered one of the chief characteristics of a resistant strain, one of two conditions must be fulfilled: either the bacterial species must be homogeneous; or the lytic principle used should have a constant activity against all types of the bacterial species. The latter necessity should be kept constantly in mind when one is dealing with the so-called heterogeneous groups. A heterogeneous bacterial species has been denied by d'Herelle[1] as one in which certain strains are nonsusceptible to bacteriophage activity while others are attacked. But the susceptible strains will differ when various bacteriophage strains are used. He states that this natural immunity is limited, not absolute; so that one would expect the evidence of resistance to be a difficulty in lysing but seldom a failure to lyse, if a sufficiently polyvalent filtrate is available for these heterogeneous groups. Bacillus coli is probably the best illustration of a heterogeneous species. D'Herelle calls it the "most heterogeneous of all bacterial species." This, he states, is largely because it is so frequently found as a mixed culture with the bacteriophage, as in the intestinal tract.

A recent detailed article on the bacteriophage in urinary infections illustrates the fallacy of drawing conclusions concerning the frequency of resistant strains of B. coli. Larkum,[2] using a single anticolon bacteriophage finds only 25% of the strains from 109 urines, susceptible; while with sewage filtrate the author in a short series recently undertaken, finds 95% of the organisms susceptible to lysis. These strains show a great variety of growth characteristics and peculiarities and vary considerably in the readiness with which

[1] The Bacteriophage and Its Behavior (translation), 1926.

[2] J. Bact., 1926, 12, p. 203.

they may be lysed so that there is, doubtless, a high percentage of resistant strains among them. But resistance must be established on the basis of more dependable observations than absence of lysis by a bacteriophage of limited potency. Resistance will, rather, evidence itself in modified characteristics as suggested by d'Herelle,[1] Hadley,[3] Dutton[4] and others. As these criteria have not been fully worked out or established to be the same for all bacterial species, it seems important to the author that the obtaining of a lytic principle of maximum polyvalence be the first consideration of those who wish to classify organisms into resistant and other groups.

Experiments now in progress indicate a definite value of sewage filtrate as a source of bacteriophage for the treatment of urinary infections. Practically all writers on this subject agree that, when lysis of the causative organism occurs in vitro, clinical and bacteriological improvement usually occurs. Bacillus coli is found in the majority of these infections and the chief obstacle to the use of the bacteriophage in treatment has been the fact that so many cultures are resistant to lysis by the few strains of anticolon bacteriophage available to the investigators. The use of sewage filtrate as a source of bacteriophage should materially increase the number of cases which can be treated successfully and quickly.

It has been noted by a number of observers [1,5] that sewage-contaminated water such as rivers and harbors contains active bacteriophage. The organisms tested were usually single strains of Bacillus typhosus, dysenteriae, and coli. Hadley[6] has found the filtrate of sewage active against B. typhosus. With a sterile filtrate of affluent city sewage I have obtained lysis with a variety of pathogenic organisms, all from human diseases. The series is not a complete study of the possibilities of sewage filtrate and deals chiefly with gram-negative bacilli.

Affluent city sewage is filtered twice through Berkefeld filters. The resulting fluid is clear, colorless and usually without odor. It is kept stored in 25 cc. amounts in sterile test tubes in the icebox after incubation for sterility. Some samples have been used after an interval of two months and were still as active as when fresh. Following the usual method of d'Herelle,[1] 2, 10 and 30 drops of filtrate are added to a light suspension of young organisms. The organisms tested are grouped as follows:

Typhoid and Dysentery Bacilli.—Thirteen strains of B. typhosus, isolated from typhoid patients in this laboratory; 4 stock strains of

[3] J. Infect. Dis., 1925, 37, p. 35.
[4] Ibid., 1926, 39, p. 48.
[5] Arnold, L.: Am. J. Pub. Health, 1925, 15, p. 950. Arloing, F.: Compt. rend. Soc. de biol., 1926, 94, pp. 191, 428. Bilouet, V.: Ibid., p. 708.
[6] Personal communication.

B. dysenteriae, Shiga and Flexner; and one stock strain each of B. paratyphosus A and B were used. Lysis occurred in all 19 strains on the first passage with sewage filtrate, showing either the maximum activity or activity only slightly less than maximum ($+++$ or $++++$ reaction of d'Herelle). This same group of organisms has been tested with 22 active antityphoid filtrates of the stools and urines of typhoid patients and although each filtrate was active against practically all typhoid and dysentery strains, the potency averaged much lower, and the maximum activity occurred only occasionally. Lysis of two dysentery and one typhoid strain of this group resulted with only a few of these 22 filtrates, indicating a low virulence, while sewage filtrate gave maximum lysis with the same three organisms. Thus, sewage filtrate was found to be a much better source of virulent antityphoid and antidysentery bacteriophage than the excreta of typhoid patients.

B. Pyocyaneus.—Eight strains of B. pyocyaneus isolated from urines and pus were found to be readily lysed. These strains vary considerably in pigment producing power. Some do not produce the silver metallic sheen but the majority do.

Other Gram-Negative Bacilli.—Forty strains of gram-negative bacilli isolated from catheterized specimens in urinary infections were lysed by sewage filtrate. There is a greater variation in this group in the readiness with which lysis occurs but, in a little over half, lysis was marked ($+++$ or $++++$) in the first passage and with the others only three or four passages were necessary to enhance the principle to this point. In this study of urinary infections, only cases showing a pure culture of a gram-negative bacillus were used and sewage filtrate has failed to lyse only one organism in the first consecutive 27 cases. The other 13 cases were taken at random from urinary infections before the routine study was begun and were selected because they showed unusual growth characteristics, or plaques, on the isolation plates. All but one of these organisms were lysed by sewage filtrate. The majority of these 40 gram-negative bacilli are B. coli but a few belong to the mucosus capsulatus group.

Sewage filtrate has so far failed to lyse a few gram-negative bacilli, even after five serial passages. Aside from the two mentioned in the urinary infection group, 6 other bacilli have proved resistant. These were obtained in blood cultures from patients with conditions simulating low grade endocarditis, by Rosenow's concentration method. These 8 resistant bacilli are all peculiar organisms; 3 produce a diffusing amber pigment 2 others a slight yellowish pigment. These organisms

are not the usual contaminants and are being studied as to their possible rôle as bacteriophage carriers.

Gram-Positive Cocci.—Of 8 streptococci tested with as many as 4 passages, 3 were lysed on the second or third passage with sewage filtrate. Two of these were from the blood stream of patients with subacute bacterial endocarditis. The other was from a urinary infection.

Of 8 staphylococci tested, 2 were lysed with sewage filtrate. These results suggest that sewage filtrate will furnish a virulent bacteriophage for enough gram-positive cocci so that it is worth testing when such a bacteriophage is desired. A much more exhaustive study is needed with this group.

CONCLUSIONS

Sewage filtrate as tested with 67 strains of gram-negative organisms yields a potent bacteriophage for practically all strains of Bacillus coli isolated from urinary infections, and for most of the other common gram-negative bacilli encountered in human diseases.

The use of sewage filtrate as a source of bacteriophage will materially increase the number of urinary infections that can be treated with the bacteriophage; and will avoid confusion in the identification of resistant strains of bacteria.

THE PERSISTENCE OF BOTULINUM TOXIN IN DISCARDED CANNED FOODS[*]

WILLIAM A. STARIN

From the Department of Hygiene and Bacteriology of the University of Chicago

A review of the literature reveals a number of reports of experiments conducted to determine the stability of the toxin of Cl. botulinum in its relation to various external factors. In common with other experimental work with Cl. botulinum and its toxins, the lack of agreement in the findings is quite evident.

Van Ermengem,[1] in his papers reporting the earliest work on botulism, states that the toxin of Cl. botulinum is easily destroyed by the action of air and light, but that when kept in the dark, deterioration progresses slowly, some degree of toxicity being evident for months. Flesh appeared to preserve the toxin, since pieces of meat kept in the dark in test tubes remained toxic for considerable time. Putrefaction of meat, blood, feces, and urine did not result in detoxification within four days. Brieger and Kempner,[2] working with purified toxins obtained by precipitation of the cultures with $ZnCl_2$ or $(NH_4)_2SO_4$, report that such products, dried at ordinary atmospheric temperatures, retain their toxicity with only slight impairment, provided care is taken to prevent an increase in the acidity since any such increase is inimical to the stability of the toxin. Landmann,[3] in connection with his investigation of the outbreak of botulism at Darmstadt, stated that the toxin is readily destroyed by air, heat, and light. Schübel[4] evaporated the toxic medium to dryness in vacuo, at 20 to 25 C., and found that the dry product obtained was still actively toxic, and that it remained so for a considerable time.

Bronfenbrenner and Schlesinger[5] report that the toxin of Cl. botulinum is resistant to the proteolytic action of pepsin and trypsin. Thom, Edmondson and Giltner[6] studied the effect of light and temperature on the persistence of the toxin, and found that while temperatures of 68 to 75 C. destroyed it within ten minutes, ordinary atmospheric temperatures exerted little influence if the materials were kept in the dark. Under such conditions, the toxin could still be detected after two months. Stored in test tubes of ordinary glass, complete destruction of the toxin had not taken place after forty hours' exposure to the direct action of sunlight. Bitter,[7] in his summary on botulism, concludes that the toxin is susceptible to light but that the rate of destruction is gradual. Schoenholz and Meyer[8] studied the action of light and temperature on the toxin. They placed toxin produced in broth cultures and in vegetables, in test

[1] Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 1; Handbuch der pathogen, Mikroorganismen, 1912, 4, p. 909.

[2] Deutsch. med. Wchnschr., 1897, 23, p. 521.

[3] Hyg. Rundschau, 1904, 14, p. 449.

[4] Deutsch. med. Wchnschr., 1921, 47, p. 1047.

[5] Science, 1921, 54, p. 444; J. Exper. Med., 1924, 39, p. 509.

[6] J. Am. M. A., 1919, 73, p. 907.

[7] Ergebn. d. allg. Path. u. path. Anat., 1921, 19, pt. 2, p. 733.

[8] J. Infect. Dis., 1924, 35, p. 361.

tubes and brown bottles of ordinary glass, and exposed these to direct and to
diffuse light.   Under these conditions, toxins could be detected after four
months' exposure to diffuse light.   Toxic hay, dried and exposed to direct
sunlight, remained toxic for nineteen days.   In her monograph, "Studies of
Organisms concerned as Causative Factors in Botulism," Bengtson[9] discusses
these variable findings, and points out different combinations of factors that
may have contributed to these contradictory results.   Her own experimenal
work was concerned with the relative persistence of the toxicity in fluid toxin
and in toxic material prepared by the precipitation of toxic broth cultures with
$(NH_4)_2SO_4$ and dried in a manner analogous to the procedure used in the prep-
aration of purified tetanus toxin.   Her findings indicate that both types of toxic
products thus artificially produced and kept under standard conditions as
regards air, light, and temperature, exhibit a high degree of persistence.

A survey of the literature cited shows first, that markedly varying
results have been reported; and second, that with a few exceptions, the
reported findings have been based upon a study of toxins produced by
the growth of Cl. botulinum in pure cultures in the ordinary laboratory
mediums.   The conditions thus encountered are not comparable to those
one finds in remnants of canned food products discarded in the ordinary
manner of the consumer.   Even in the few instances where the per-
sistence of the toxin was studied as it occurs in vegetables and meats, the
conditions of exposure were those of the laboratory rather than those of
garbage.   While marked variations exist, the prevailing observation noted
by all workers with botulinum toxin is that the toxin resulting from
cultivation of pure cultures in artificial mediums undergoes a rapid initial
deterioration, after which it becomes relatively stable, and the subsequent
decrease in toxicity is slow and gradual.

It was considered desirable to undertake a series of experiments in
which the persistence of the toxin would be studied, not as it is produced
under artificial conditions in the laboratory, but as it is encountered in
discarded canned goods in which the organism had developed and pro-
duced its toxin.   This was considered desirable both from the academic
standpoint of acquiring knowledge relative to the stability of the toxin
under such conditions, and also because such experiments might throw
some light on the practical problem of determining for how long a time
one might reasonably expect to find demonstrable toxin present in
canned foods as ordinarily discarded by the average housewife.   These
findings might be of value in determining the character of outbreaks of
food poisoning, and the sources of the materials responsible.

Procedure.—Organisms: Strains of both the A and B types of Cl. botulinum
were used in the experiments.   A representative culture of type A was used.
This strain came from a single cell isolation of a culture originally obtained

    [9] U. S. P. H. S., Hyg. Lab. Bull., 1924, No. 136, p. 49.

from the olives responsible for the Greensburg, Pa., outbreak in 1921. It produces regularly a toxin of extremely high potency—beef heart cultures in quantities as low as 0.000,005 cc. killing white mice when introduced intraperitoneally. The B type used was a single cell culture originally isolated from grain by Dr. Graham of the University of Illinois. While it produces the characteristic toxin regularly, the potency is low, the average MLD for white mice being 0.001 cc. introduced peritoneally.

The organisms used for the inoculations were cultivated in beef heart for ten days at 37 C., after which time they were found to consist almost entirely of free spores. These spore suspensions were washed free of medium by repeated centrifuging in physiologic salt solution, and were then heated at 80 C. for twenty minutes, to detoxify them. Tests of the efficacy of the heating to render the material atoxic were carried on by injections into white mice. The atoxic suspensions were then standardized for spore content by direct count and colony counts, and the different cans of material each injected with a constant inoculum. This consisted of 500,000,000 detoxified spores per number 2 can.

Mediums: The vegetables used were peas and corn, and salmon was selected as a typical animal protein substance. All were commercially canned in number 2 containers. Inoculations of the cans were made using all precautions ordinarily employed to avoid contamination. The material was tested for sterility prior to the inoculations. Following inoculation the cans were resealed and incubated at 37 C. for two weeks. They were then examined and were found to contain potent specific toxins. The MLD of the toxin produced by the A strain in the different mediums varied from 0.00005 cc. to 0.000,005 cc. The MLD of the toxin produced by the B strain under the same conditions was on an average 0.001 cc. White mice served as test animals and injections were all intraperitoneal.

Five-gram quantities of the different mediums were removed from the various containers and were smeared over the inner surfaces of ordinary number 1 clean and sterilized cans. Care was taken in spreading the material to avoid the presence of lumps or masses of medium, the object being to spread it in such a manner as to duplicate ordinary conditions in discarded cans. The quantity selected was considered typical of the amount ordinarily found in such discarded tins. With the exception of the cans to be kept out of doors all were left uncovered. Those comprising the out-of-doors series were covered with a double layer of cheesecloth to prevent the entrance of gross particles of dust.

Conditions of exposure: The toxic material was exposed to three different combinations of environmental conditions. A number of samples of each medium, inoculated with each organism, were placed in dark lockers at room temperature; other samples were exposed at room temperature to diffuse and bright daylight, though not to direct sunlight; still other samples were exposed to the variable out-of-door temperatures of the season in which the test was made, but were kept covered to prevent the action of light; a final set of the toxic material was exposed to outdoor temperatures, to direct light, wind, and rain, although sheltered to prevent the entrance of water directly into the can.

Series: Two series of experiments were set up to study the climatic effects upon the persistence of the toxin. The first series was commenced on April 24, and observations and tests were made periodically until August 1, making a period of 99 days. The second series was begun June 20 and the final observations and tests were made August 28, making a period of 68 days. No attempt was made to record the daily temperatures, amount of direct sunlight, or rainfall, since they were variable but agreed very closely with the mean for the

respective seasons of the year. One set was thus exposed to conditions of moderate heat, light, and rainfall, as would be experienced in the spring months; the other was subjected to summer climatic conditions.

Series 1.—Tests were made at intervals of one week during the early part of the first series. After finding the persistence greater than we had anticipated, the testing time was extended to ten day intervals. This plan was employed also in series 2.

The method of testing consisted in weighing carefully each can, adding a definite quantity of sterile distilled water which was allowed to extract the macerated material for three to five hours in the can, and then subjecting the material to grinding in sterile mortars to liberate any toxin within the small

TABLE 1

PERSISTENCE OF BOTULINUM A AND B TOXINS IN DISCARDED CANNED FOODS SHOWN BY EFFECT ON MICE

	Mouse	Medium	Condition, Temperature, Light		P_H	Maximum Dilution	Results, Death, Hours after Injection
Each mouse given 0.25 cc. botulinum A toxin	1	Peas	Room,	Light	6.1	1:20,000	24
	2	Peas	Room,	Dark	6.2	1:25,000	24
	3	Peas	Outdoor,	Light	6.3	1:10,000	48
	4	Peas	Outdoor,	Dark	6.4	1:20,000	24
	5	Corn	Room,	Light	5.2	1:20,000	24
	6	Corn	Room,	Dark	5.1	1:25,000	24
	7	Corn	Outdoor,	Light	4.9	1:10,000	48
	8	Corn	Outdoor,	Dark	5.2	1:25,000	48
	9	Salmon	Room,	Light	7.1	1:5,000	24
	10	Salmon	Room,	Dark	7.2	1:10,000	24
	11	Salmon	Outdoor,	Light	7.2	1:5,000	24
	12	Salmon	Outdoor,	Dark	7.1	1:10,000	24
Each mouse given 0.25 cc. botulinum B toxin	13	Peas	Room,	Light	6.5	1:500	48
	14	Peas	Room,	Dark	6.3	1:500	24
	15	Peas	Outdoor,	Light	6.5	1:250	48
	16	Peas	Outdoor,	Dark	6.5	1:500	24
	17	Corn	Room,	Light	5.1	1:1,000	24
	18	Corn	Room,	Dark	5.2	1:1,000	24
	19	Corn	Outdoor,	Light	5.1	1:350	48
	20	Corn	Outdoor,	Dark	5.2	1:500	48
	21	Salmon	Room,	Light	—	1:500	24
	22	Salmon	Room,	Dark	—	1:500	48
	23	Salmon	Outdoor,	Light	—	1:500	48
	24	Salmon	Outdoor,	Dark	—	1:250	48

masses. The emptied cans were washed thoroughly and dried over an oven. The dry cans were then reweighed and the amount of solid material they had contained was determined. From these weighings the dilution of the toxin to be used for injections was calculated. The dilutions were made from these extractions, using sterile distilled water. The P_H was determined each time to note any changes in reaction.

Each sample of food inoculated with the different organisms was tested for toxicity by making intraperitoneal injections into white mice. Three dilutions were made with each sample. Table 1 summarizes the findings of one test in series 1, run at the end of a period of 99 days. These findings are typical of the results obtained in all the experiments. Antitoxin controls were run on each toxic sample—with complete protection where homologous antiserum was used.

Series 2.—This series which was carried out in a similar manner, excepting that the exposure time was from June 20 to August 28, yielded results that were almost identical to those in series 1. The time of exposure in this instance was much shorter, being 68 days, but the exposure was that of an average summer as regards heat, light, and moisture. In every instance toxin could be demonstrated in lethal doses, in dilutions 1 : 5,000 to 1 : 10,000 of the A strain, and in dilutions 1 : 250 to 1 : 1,000 of the B strain. Comparing this with the original titer of the material, one notes a decrease in toxicity of the B type, but that of the A type maintained itself with only slight diminution.

The physical appearance of the mediums varied with the conditions of exposure. The cans kept in the dark, both indoors and out of doors, retained their natural color and odor. During the period of exposure, the five-gram quantities decreased in weight to less than one gram. The mediums exposed to the light in doors showed much more discoloration and drying, being almost horny in consistency. The weight decreased to 0.6 to 0.8 grams. The material exposed to the light and weather out of doors was covered with dust and dried down almost to a powder. On account of the collected dirt, exact weight could not be obtained. The appearance suggested the impossibility of toxin retention, and had such material been submitted to the average diagnostic laboratory, it would have been rejected.

Series 3.—A third series of experiments was conducted, with the object of studying the effects of prolonged exposure of toxic materials to air, light, and temperature without, however, having been subjected to drying. The original cans of peas, corn, and salmon, small portions of which had been used in series 1 and 2, served as the material for this set of experiments.

The cans, which contained approximately 350 grams of toxic material, were left open, exposed to diffuse light, air, and the varying temperatures from April 24 to August 28 (122 days). Examination of the contents at the end of this period revealed evidence of marked bacterial contaminations. Putrefaction was far advanced in all the containers. Small weighed quantities of this putrid material were ground with sterile sand and distilled water, in sterile mortars, and then passed through Berkefeld filters. The sterile filtrates were divided into three portions. One fraction was mixed with its homologous antitoxin; one was heated at 75 C. for thirty minutes to destroy any botulinum toxin and to serve as test material for other thermostable toxins that might be present; the third fraction was used untreated. Intraperitoneal injections of varying dilutions of each of the above fractions were made into white mice. The animals receiving the injections of the untreated fraction in dilutions as high as 1 :5,000 died in forty-eight hours, with typical symptoms of botulism. The mice receiving the heated fraction and the mixture of the filtrate and antitoxin remained normal.

The results of these experiments seem to show that the botulinum toxin is stable in the presence of putrefaction, exposure to light, and a summer temperature, when kept in a moist state.

Series 4.—While it did not seem probable, the question arose as to the possibility of the Cl. botulinum spores growing in the material used in series 1 and 2 during the period of exposure. Were this true, the apparent persistence of the toxin would in reality be due to the presence of newly formed toxin produced by the metabolism of the organisms themselves. To answer this question, small quantities of sterile peas, corn, and salmon, were inoculated heavily with detoxified Cl. botulinum spores, and then similarly exposed to the same environmental factors present in series 1 and 2. These specimens were tested for botulinum toxin at weekly intervals over a period of 35 days. While viable organisms could always be recovered, no indications of toxin production were ever observed. Thus the toxin demonstrated in series 1 and 2 was that originally present and could not be ascribed to any production during the period of exposure.

The practical significance of this stability of the toxin is evident. Although negative findings cannot be accepted as conclusive, it is reasonable to expect to find demonstrable toxin in discarded vegetables and meats, if they were originally toxic, even after relatively long periods of time. The fact that materials submitted for examination may reach the laboratory in a dried condition does not preclude the possibility of demonstrable botulinum toxin.

CONCLUSIONS

Both the A and B types of Cl. botulinum are capable of producing toxin in corn, peas, and salmon, that will withstand exposure to drying, light—both diffuse and direct—and seasonal variations of temperature for a period of at least 90 days.

The toxin present after such prolonged exposure is the persisting original toxin, and is not newly formed as the result of the metabolism of Cl. botulinum that may have been present in the original material, or that may have been introduced subsequently as a contaminant.

Putrefaction of vegetables and meats which were originally highly toxic does not cause a disappearance of the toxin. Peas, corn, and salmon, exposed in a moist condition and highly contaminated with putrefactive organisms, yielded botulinum toxin after an interval of one hundred and twenty-two days.

IMPERMEABILITY OF THE SMALL INTESTINE OF RABBITS TO BOTULINUM TOXIN *

GAIL M. DACK AND WILLIARD L. WOOD

From the Department of Hygiene and Bacteriology, University of Chicago

In a previous publication [1] the permeability of the small intestine to botulinum toxin was tested using rabbits and hogs. In those experiments an artificial circulation of blood was established by means of a Woodyatt pump.[2] The object of these experiments was to make a similar study under better physiologic conditions.

Type A toxin used in these experiments was produced by growing Cl. botulinum in tubes of beef heart medium for ten days under a vaseline seal. The supernatant fluid from these cultures was centrifuged at high speed for one hour which removed most of the organisms.

The rabbit was given an injection with 0.1 gm. of heparin in 5 cc. of sterile (0.85%) salt solution and placed under ether anesthesia. The small intestine was exposed and placed in warm gauze packs which were kept moist with warm salt (0.85%) solution. A suitable loop of small intestine was selected and the vein coming from it was cannulated, but the artery was left intact. The loop was then carefully ligated and the toxin injected in the end nearest the stomach. The point of injection was touched with tincture of ferric chloride and this portion of the loop was ligated. The loop was carefully manipulated so as to distribute the toxin throughout its length. The animal was kept alive by frequent blood transfusions, using heparinized blood from normal rabbits. Samples of blood were collected at five minute intervals and injected into mice in 0.5 and 0.1 cc. amounts. At frequent intervals, also, mice were given injections with 0.5 cc. of blood and 0.25 cc. of the homologous antitoxin.

Exper. 1.—A loop of intestine one foot long, three inches from the pylorus of the stomach and six and one-half feet from the ileo-cecal valve was chosen. The loop appeared normal and showed motility at the end of the experiment. Four cc. of toxin were injected into the loop. There was some leakage of toxin from the plunger of the syringe. This toxin was caught on a towel which was immediately removed and the portion of the animal (thoracic) covered by it was washed off with alcohol. One-half cc. of a 1:5,000 dilution of toxin injected intraperitoneally into a mouse killed the mouse within twenty-four hours. Dilutions higher than this were not made.

Four and one-half cc. of contents were expressed from the loop and the P_H was found to be 8.5. This material was diluted with sterile 0.85% salt solution and centrifuged at high speed for one hour. One-half cc. of a dilution of 1:10,000 produced symptoms of botulism when injected into a

white mouse, while a dose of 0.5 cc. of a 1:5,000 dilution caused death in one day. Mice receiving 0.5 cc. amounts of lower dilutions of the centrifuged supernatant fluid plus 0.25 cc. of the homologous antitoxin lived. One-half cc. of heart blood taken from the animal at the close of the experiment and injected into a mouse produced death in 2 days, while a mouse receiving the same amount plus 0.25 cc. of homologous antitoxin lived. A mouse receiving 0.1 cc. of the blood lived (table 1).

Exper. 2.—A loop of intestine eight inches long, five feet from the pylorus of the stomach and three feet from the ileocecal valve was chosen. The loop appeared normal and showed motility at the end of the experiment. One cc. of toxin with an MLD of 0.001 cc. was injected into the loop and at the end

TABLE 1

Permeability of the Duodenum of Rabbit to the Toxin of Cl. Botulinum, Shown by Toxic Effect of the Rabbit Blood on Mice

Intervals (Each 5 Minutes)	Cc. of Blood Transfused from Normal Rabbits	Cc. of Blood Collected from Treated Rabbit	Toxin in 0.5 Cc. Blood* (by Mouse Injection)
0	—	8.0	0
1	—	3.25	0
2	12	13.0	0
3	12	14.0	0
4	13	14.0	0
5	—	9.5	0
6	10	6.0	0
7	10	10.0	0
8	11	12.0	0
9	13	10.5	0
10	11	8.0	0
11	8	4.0	0
12	—	7.0	+
13	—	8.0	+
14	—	5.0	+
15	—	3.0	+
16	20	2.0	+
17	—	4.0	+
18	—	12.0	+
19	—	4.25	+
20	—	3.0	+
21	—	1.25	+
22	—	1.25	+
Total 1 hour and 50 minutes	130	163.00	

* Mice receiving 0.1 cc. portions lived.

of one hour and fifteen minutes, 1.5 cc. of intestinal contents were removed. This mixture was diluted in sterile 0.85% saline solution and 0.5 cc. of a 1:1,000 dilution when injected into a mouse caused death in one day. A mouse injected with 0.5 cc. of a 1:5,000 dilution showed symptoms but did not die. Mice receiving 0.5 cc. amounts of lower dilutions of the centrifuged supernatant fluid plus 0.25 cc. of the homologous antitoxin lived. One-half cc. of the heart blood taken from the animal at the close of the experiment and injected into a mouse failed to produce symptoms. With but one exception the mice receiving 0.5 cc. or 0.1 cc. amounts of the blood drawn at the five minute intervals failed to show symptoms. The exception was the mouse receiving 0.5 cc. of blood taken at the first five minute interval. This mouse showed symptoms but lived.

DISCUSSION

In experiment 1. no toxin was found in the blood stream until 60 minutes had elapsed. The source of the toxin which entered the blood stream is not known. The heart blood was toxic at the close of the experiment, although no more toxin was found in it than in the blood from the loop, which would indicate that the source of the toxin was not from the isolated portion of intestine. It may have entered through the lymphatics or diffused through the tied off portion of intestine at the point of injection or through faulty technic in handling the toxin during the injection.

The volume of blood collected increased immediately after each transfusion. The first transfusion in each case seemed to cause an initial vasodilation which persisted throughout the experiment.

In experiment 2 the toxin introduced into the loop was observed to have increased in potency. The volume of the loop contents in both cases was small, showing no abnormal distention. Distention of the intestine may be of considerable importance in the absorption of botulinum toxin if the toxin behaves as do some protein substances.[3]

CONCLUSIONS

The duodenum and ileum of the rabbit were found not to be permeable to botulinum toxin under the conditions of the above experiments. Some toxin was demonstrated in the blood from the duodenum after 60 minutes but not more than was found in the heart blood at the end of the experiment.

[3] Hettwer, J. P., and Kriz-Hettwer: Am. J. Physiol., 1926, 73, p. 136.

THE ISOLATION, PURIFICATION AND CHEMICAL NATURE OF IMMUNE HEMOLYSINS

Susuma Uchida

From the Research Institute of Cutaneous Medicine, Philadelphia

The isolation and purification of antibodies for the purpose of determining their chemical nature and improving serum therapy, have been for many years among the most interesting problems in immunology.

Since 1905 a rather extensive literature has accumulated upon the subject which it is hardly profitable to review here because it is obvious that many of the methods employed have not been successful in isolating antibody in sufficient purity for chemical study. Precipitation of immune serum with ammonium sulphate for example, has shown that antibodies are carried down in the globulin fractions but it is generally granted that this does not necessarily indicate that antibodies are globulins. Adsorption of antibody by such substances as kaolin and charcoal followed by attempts at dissociation have also failed as likewise alternate freezing and thawing of sensitized antigen. Insofar as the hemolysins are concerned special mention may be made of the work of Von Liebermann and Fenyvessy,[1] who claimed success in dissociating anti-guinea-pig hemolysin from sensitized corpuscles by means of treatment with hydrochloric acid, and of Rondoni[2] who used sodium hydroxide. More encouraging results have been reported by Kosakai[3] with isotonic and hypertonic solutions of various sugars for dissociating hemolysins from sensitized cells although he has not reported upon the results of any chemical studies with the isolated antibodies. Huntoon[4] and his associates have met with so much success in the isolation of pneumococcus antibody that I have applied his methods and those of Kosakai in further attempt to isolate and purify antisheep and antihuman hemolysins for chemical analysis. The results are briefly summarized in this communication.

Ottenberg[5] has endeavored to isolate the hemolysins by precipitating immune serum with the chlorides and sulphates of some of the heavy metals followed by dissolving out the antibody from the precipitates, but on account of the large amount of protein present was unable to isolate them in a pure state.

Locke and Hirsch[6] however, by a process of electrodialysis unassisted by isoelectric fractionation, have succeeded in isolating from serum the immune isoelectric pseudoglobulin in such purity that each hemolytic unit contained but 0.000,002 gm. of protein and some lipin. They also recovered hemolysin from sensitized corpuscles through a destruction of their combining capacity by means of extraction with ether, which partially removes the lipins of the stroma correlated with a shift of the isoelectric point of the stroma from

[1] Centralbl. f. Bakteriol., 1, O., 1908, 47, p. 274.
[2] Ztschr. f. Immunitätsforsch. u. Exper. Therap., 1910, 7, p. 515.
[3] J. Immunol., 1918, 3, p. 109.
[4] Ibid., 1920, 4, p. 117.
[5] Proc. Soc. Exper. Biol. & Med., 1923-1924, 21, p. 14.
[6] J. Infect. Dis., 1924, 35, p. 519; 1925, 37, p. 448; 1926, 39, p. 126.

approximately P_H 5 to P_H 7, and yielding hemolysin of such purity that but 0.000,125 to 0.000,18 mg. of protein was associated with each hemolytic unit.

General Technic.—Antisheep hemolysins were prepared by the immunization of rabbits with intravenous injections of 5 cc. of 10% suspensions of washed corpuscles every five days for five injections (Kolmer method) which generally resulted in the production of immune serums possessing a hemolytic unit of 1:8000 when titrated with 1 cc. of 2% corpuscle suspension and 1 cc. of 1:20 guinea-pig complement. As usual the antihuman hemolysins were much more difficult to prepare and the intravenous injection of rabbits with 0.1 cc. of sediment of washed corpuscles each day for three weeks (Thompson method) generally resulted in producing serums with titers of 1:60 to 1:80 when titrated with 1 cc. of 1% corpuscle suspension and 1 cc. of 1:10 complement.

Sheep corpuscles were sensitized by adding 10 cc. of washed cells to 100 cc. of 1:100 hemolysin followed by incubation at 38 C. for two hours. The corpuscles were then washed five or more times to remove all trace of protein. Human corpuscles were sensitized by adding 10 cc. of washed cells to 50 cc. of 1:10 hemolysin followed by similar incubation and washing. Tests of the supernatant immune serums showed complete absorption of the respective hemolysins in every experiment.

TABLE 1

DISSOCIATION OF ANTIBODIES.

Extracts	Hemolysins				Hemagglutinins	
	Antisheep Serums		Antihuman Serums		Antihuman Serums	
	(1:8000)	(1:10,000)	(1:80)	(1:64)	(1:1280)	(1:640)
Saccharose.............	1:10	1:10	1:2	0	1:8	1:6
Dextrose................	1:4	1:8	1:2	0	1:6	1:5
Sodium chloride........	0	0	0	0	1:2	1:2
Sodium bicarbonate....	0	0)	0	0	1:8	1:8

Unit of hemolysin, and agglutinin titer of whole serum indicated by figures ( ) in column headings.

For dissociation 2 cc. of the washed sensitized corpuscles were added to 18 cc. of each of the following solutions: (a) 10% saccharose; (b) 5.6% dextrose; (c) 0.85% sodium chloride and (d) 0.5% sodium bicarbonate in 0.85% sodium chloride solution. These mixtures were placed in a water bath at 55 C. for thirty minutes during which they were shaken several times and then placed in a refrigerator over night. Each mixture was then centrifugated and the supernatant fluids (all of which were tinged slightly with hemoglobin) tested for hemolysin in the same manner as the immune serums before absorption. At the same time each supernatant fluid was examined for hemagglutinins.

The results of the experiments with two antisheep and two antihuman serums are shown in table 1 as examples of the whole.

It will be noted that the degree of dissociation of the hemolysins was quite weak and occurred only in the saccharose and dextrose solutions. For example, in the method employed each cc. of washed sheep corpuscle sediment was sensitized with approximately 4000 units of hemolysin but yielded only about 50 units in the saccharose solution, 20 to 40 units in the dextrose solution and none at all in the sodium chloride and sodium

bicarbonate solutions. With the antihuman hemolysins the results were almost negligible and the whole series of experiments showed that it is apparently more difficult to dissociate these two hemolysins from sensitized corpuscles than it is to dissociate pneumococcus antibody. While the antisheep serums contained no agglutin both antihuman serums contained large amounts and it is of interest to note in table 2, the degree of their dissociation in the four solutions employed in this study.

Naturally these findings were very discouraging for the chief part of this investigation, namely, a chemical study of isolated hemolysin, because the degree of dissociation was so slight, but before taking up this subject it may be well to give attention to a few of the more important details concerning their isolation and purification.

TABLE 2

Results of Hemolytic Activity Hemolysin Solutions After Treating with Ether and Dialysis

	Degree of Hemolysis						
	Dilutions of Fluids						
Test Fluids	Undiluted	1:2	1:4	1:8	1:16	1:32	Controls
Sugar solution	Complete	Complete	Complete	Moderate	Slight	0	0
After treatment with ether	Complete	Complete	Complete	Slight	0	0	0
Before dialyzing	Complete	Complete	Complete	Moderate	Slight	0	0
After dialyzing	Complete	Complete	Moderate	Slight	0	0	0

In the first place were the saccharose, dextrose and bicarbonate solutions antihemolytic and thereby likely to mask the presence of dissociated hemolysins? The results of experiments have shown that dilutions of hemolysins prepared with them were but very slightly less hemolytic than control dilutions prepared with physiological saline solution. For example, in one experiment employing antisheep hemolysin the units were as follows:

Diluted with salt solution 1:50,000
Diluted with saccharose solution 1:30,000
Diluted with dextrose solution 1:30,000
Diluted with sodium bicarbonate solution................ 1:50,000

It would appear therefore, that the weak hemolytic activity of the solutions containing dissociated hemolysins was due to the presence of but small amounts of antibody. But repeated extractions of sensitized corpuscles have shown that all of the dissociable hemolysins are not given off in the first extraction. For example, when sheep corpuscles were sensitized and extracted with saccharose solution as described above, the first extraction fluid yielded a titer of 1:8 of dissociated

hemolysin. When the corpuscles were secured by centrifuging and extracted again the second extraction fluid showed a titer of 1:4. Third and fourth extractions still showed the dissociation of smaller amounts of hemolysin but unfortunately the corpuscles likewise became more and more fragile with the liberation of hemoglobin.

Of further importance is the question of the degree of dissociation of hemolysin in relation to the amount of dissociating solution and number of sensitized cells since both Kosakai and Huntoon have shown that the dissociating activity of sugar solutions is influenced by the quantity employed as influencing the dilution of electrolyte. Likewise the degree of sensitization of the corpuscles in relation to the degree of dissociation of hemolysin in constant amounts of sugar solution is of interest in this connection.

My experiments have shown that the extraction of 2 cc. of washed sensitized sheep corpuscles with 35 cc. of saccharose solution brought about a slightly greater degree of hemolysin dissociation than extraction with 10 cc. of the solution; but extractions with 5 to 20 cc. of sugar solution yielded almost identical results. It should be stated in this connection however, that the larger amounts of the sugar solution also resulted in more injury to the corpuscles than the smaller amounts, as shown by the release of more hemoglobin. But variations in the degree of sensitization of the corpuscles had almost no effect upon the degree of dissociation in saccharose solution, probably because the cells were well saturated with hemolysin. For example, sheep corpuscles sensitized with 5 cc. of 1:100 hemolysin yielded up as much dissociated hemolysin as an equal number of corpuscles sensitized with 20 cc. of the same hemolysin at the same time and in the same manner.

The temperature of the dissociating fluid, however, has an important bearing on the degree of dissociation of hemolysin. Kosakai found higher temperature more favorable than low temperature; Rondoni however, observed just as good dissociation in weak alkali solution at 0 C. as at 37 C. Huntoon observed but very little influence of temperatures from 42 C. to 55 C. upon the dissociation of pneumococcus antibody in distilled water and salt solution. In my experiments with sensitized sheep corpuscles, dissociation in saccharose solution was most active at temperatures varying from 37 C. to 55 C. Temperatures higher than 55 C. also caused changes in the corpuscles and increased hemoglobin release. However, it may be stated in passing, that the dissociation of typhoid agglutinin and complement fixing antibody occurred best at temperatures of 60 C. to 65 C. as separately reported.

Of additional interest is the influence of time on the degree of dissociation of hemolysin. Employing sheep corpuscles sensitized as previously described and each 2 cc. of washed cells extracted with 20 cc. of 10% sacchararose solution, I observed that the degree of dissociation at 55 C. was just as good at the end of thirty minutes as at the end of two hours; indeed the amounts of free hemolysin at intervals of 30 to 90 minutes were just a little greater than at the end of the two hour period.

Finally special mention may be made of dissociation of the hemolysins by saccharose solution rendered faintly alkaline with sodium hydroxide since Furuhata [7] observed that the addition of alkali or acid to saccharose solution appeared to increase the degree of dissociation of hemaglutinins. I have extracted 2 cc. amounts of washed sensitized sheep corpuscles under identical conditions and at the same time with 20 cc. of 10% saccharose solution and 19 cc. of saccharose solution plus 1 cc. of decinormal solution of sodium hydroxide, the latter being later neutralized before testing for dissociated hemolysin. The results have been identical and similar results were observed with sensitized human corpuscles.

The Purification and Concentration of Dissociated Hemolysin.—It is almost sure that the hemolysins dissociated in sugar solutions contained not only the antibody but also destroyed corpuscles; therefore the solutions must be purified in order to investigate the chemical nature of the hemolysins.

Elimination of Corpuscular Substances and Hemoglobin: Kosakai employed various means in order to remove these substances such as dialysis and adsorption methods, but these have given results. He succeeded, however, by using ether. Furuhata also employed ether for purifying the hemagglutinins. Previously Von Liebermann and von Fennyvessy used ether to purify the very slightly colored solution containing the immune hemolysins after acidifying the solution by adding 1/25 of hydrochloric acid.

Recently Ottenberg tried the effect of metallic salts on antibody already freed partially of protein by the method of specific absorption followed by the subsequent dissociation from appropriate cellular antigens. He states that in his hemolysin experiments the metallic salts interfered with the activity of complement so that the work has been more successful with the bacterial agglutinins.

It is stated that the immune hemolysins are not susceptible to ether or other fat solvents. A preliminary examination was conducted to

[7] Japan M. World, 1921, 1, p. 1.

determine whether or not this is true and I have found that hemolysin is neither soluble in ether nor harmed by it. A saccharose extract was mixed with 5 to 10 times its volume of pure ether and shaken until the lowest layer became quite colorless. In general the extraction was not complete with one mixture; therefore, the lowest layer of fluid was transferred into another funnel and again mixed with the same volume of fresh ether. In the course of this manipulation there appeared three layers of fluid: the upper layer of ether; the middle of a gelatinous mass which consisted of deteriorated corpuscles and hemoglobin and the lowest of the sugar solution. I have not infrequently found that the middle layer coagulated when the ether used had been less than 5 or 6 times the volume of the extract.

The extracted sugar solution was now transferred to a cylinder and the ether removed by the electric fan or vacuum; or the solution was placed in a water bath at 45 C. to accelerate evaporation. The evaporated sugar solution was then centrifuged at low speed, its original volume restored with sugar solution and its hemolytic activity determined by the usual technic. The results of one experiment are shown in table 2.

The results have shown that the absolute colorless sugar solution contained almost the same quantity of hemolysin as before treatment with ether. But during the course of similar experiments we encountered less hemolytic activity after this procedure which may have been due to technical errors, such as severe shaking or insufficient amounts of ether. However, it is conceivable that some of the hemolysins were transferred to the middle layer of solution with the corpuscular substances. We must also consider that complement is easily destroyed by ether in case the sugar solutions were not aboslutely free of this substance.

Elimination of Sugar: Since it is desirable to remove the sugar from the extraction fluid, both dialysis and salting out methods were used. The purified solutions of extracts containing hemolysin also contained in addition to sugar, small amounts of salt and other dialyzable substances.

Using a parchment bag as a dialyzing medium it was readily possible to remove these substances without much loss of hemolysin: 20 cc. of sugar extract placed in the dialyzing parchment bag with a small amount of toluene added as a preservative, was kept in running tap water for two days; 28 cc. of the watery solution was then tested for sugar by means of the saccharimeter and by Fehling's solution after hydrolysis with acid and both were negative for sugar. The chlorides were also removed as shown by the silver nitrate test. The purified watery hemolysins were now placed in a desiccator and concentrated until the amount was less than the amount of the original purified

hemolysin, the desiccator being kept in the dark to avoid the influence of light. The watery solutions were now isotonic and titrated for hemolytic activity.

The results have shown almost no loss of dissociated hemolysin by parchment dialysis and duplicate experiments conducted with collodion sacs yielded similar results. These experiments have shown therefore, that the hemolysins are not dialyzable.

Additional experiments were conducted by salting out methods employing ammonium sulphate. To 5 cc. of sugar solution containing hemolysin, 5 cc. of saturated solution of ammonium sulphate was added. The mixture was kept at room temperature for three hours until flocculation occurred. It was then centrifugated at high speed for one hour. The clear supernatant fluid was pipetted off and the sediments were emulsified in 3.5 cc. of physiologic salt solution and again centrifugated. The supernatant fluid was removed. These sediments were suspended in 3 cc. of salt solution. Titrations showed that a portion of the hemolysins were precipitated by means of half saturated ammonium sulphate solution; furthermore, the solutions contained traces of sugar, and ammonium sulphate. The euglobulin was practically insoluble in water and may be precipitated in the presence of 28 to 36% saturated ammonium sulphate solution. This pseudoglobulin, on the other hand, was soluble in water and only precipitated in the presence of 36 to 44% ammonium sulphate solution. Another experiment was undertaken to determine which produces the better precipitation. Two portions of solution in proportion of 3:7 and 4:6 were made with saturated ammonium sulphate solution. The mixture was centrifuged after standing at room temperature for two hours. The supernatant fluids were dialyzed, restored to original volume, made isotonic and examined by the usual methods. Both sediments were emulsified in 4 cc. of salt solution.

The results obtained from this experiment showed but little difference, although it would appear that the hemolysins were especially contained in the euglobulin fractions.

The Chemical Nature of Isolated Hemolysins.—This phase was one of the most difficult parts of the investigation because after purification the amounts of isolated hemolysin available for chemical tests were quite small.

Von Liebermann and von Fenyvessy employing concentrated and purified extracts of immune hemolysin, stated that they showed no protein reaction with the most sensitive tests but only a trace with the xanthoproteic or Millon's reagent; also that they did not penetrate animal membranes. Kosakai states that the hemolysin preparations isolated by him did not react to protein tests or to tests for sodium chloride and acetic acid. Furuhata working on the chemical nature of the hemagglutinins concluded that they are colloidal substances closely associated with protein substances in serum. They were not considered as belonging to proteins in the ordinary sense although they may con-

tain a certain amount of nitrogen in their constitution. In the studies of Huntoon and his co-workers upon the chemical nature of solutions containing pneumococcus antibody, they did not believe that the antibody belongs to the serum proteins.

Nitrogen Contents.—In my experiments the nitrogen content of hemolysin solutions after dialyzation was determined by the micro Kjeldahl method. The original serum was found to contain 1.4 gm. of nitrogen per 100 cc. serum, while the content of the final solution of hemolysin contained 0.0042 gm. of nitrogen per 100 cc. In other words the nitrogen content of the serum was reduced to 1/333 of the original amount.

The solutions were also examined for protein by the usual color and precipitating methods (table 3). It is to be noted that almost all the protein tests were negative although weakly positive reactions were

TABLE 3

PROTEIN REACTION WITH SOLUTIONS OF PURIFIED ANTISHEEP HEMOLYSIN

Tests	Diluted Serum (1:300)*	Final Hemolysin Solution
Nitrogen content per 100 cc.....................................	0.0046 gm.	0.0042 gm.
Biuret, xanthoprotein, Millon's, Hopkin's-Cole, sulphur, Heller's, acetic acid, sulphosalicylic acid, picricacid, and phosphorus	+	0
Ninhydrin, and phosphotungstic acid........................	+	Trace

* This dilution represents the same nitrogen content as the final solution.

observed with ninhydrin and phosphotungstic acid indicating that the solution probably contained a very minute amount of protein.

Effect of Trypsin on Hemolysin.—From the standpoint that immune substances may be nonprotein in nature many attempts have been made to determine the effects of trypsin digestibility. Thus Huntoon and others observed that isolated pneumococcus antibodies are not attacked by trypsin over considerable periods of time. My previous experiments showed that the isolated hemolysins are not dialyzable so they may be composed of large molecules resembling those of the globulins. If the hemolysin itself is protein, destruction of the protein by enzymes should impair the hemolytic activity of solutions and experiments were carried out with this thought in mind.

In the first place the activity of tryptic digestion upon casein was determined by using 0.1% trypsin (Digestive Ferments Co.), 0.5% casein solution and acetic acid. It was found that 2 cc. of 0.2% casein was digested by 0.5 cc. of a 0.1% trypsin. An experiment with isolated antisheep hemolysin was now conducted by arranging two sets as follows: a trypsinized extract of hemolysin in amount of 5 cc. containing 0.5 cc. of 0.1% trypsin and 0.05 cc. toluol; and a control

extract of hemolysin in amount of 5 cc. containing 0.5 cc. of salt solution and
0.05 toluol. Both were incubated for 18 hours at 37 C. and a portion of each
examined for hemolytic activity. The results were negative; that is, the tryp-
sinized extract possessed the same hemolytic activity as the control.

Another portion of each extract was now taken and an acid-alcohol
precipitating agent added. The control tube showed opalescence and
slight flocculation, while the trypsinized tube showed very slight
opalescence and no flocculation. In these experiments it was thought
that the isolated antisheep hemolysin was not destroyed by trypsin but
further investigation would be required to determine this more definitely.

*The Relation Between the Anaphylactic Reaction and Isolated Hemo-
lysin.*—For example it was important to determine if guinea-pigs could
be sensitized with solutions of purified hemolysin. If the immune hemo-
lysin is associated with serum protein or if it is a protein itself, it may
be possible to sensitize these animals and the following experiments were
performed to determine this. It would appear that the hemolysin solu-
tions were unable to sensitize guinea-pigs to rabbit serum although they
contained minute amounts of nitrogen.

Three guinea-pigs of about 300 gms. in weight were selected. One was
employed as the control; 5 cc. of the solution bearing hemolysin were injected
intraperitoneally into the other two. Into the control animal 0.01 cc. of fresh
normal rabbit serum was injected by the same route. After 14 days the injection
of normal rabbit serum was made intravenously into each guinea-pig. The
control animal showed the usual symptoms and signs of anaphylaxis whereas
the two animals injected with the hemolysin solution did not.

SUMMARY

A slight degree of dissociation of immune hemolysin from sensitized
sheep and human erythrocytes was found to occur in 10% solution of
saccharose and in 5.6% solution of dextrose in the first extraction, and
additional but smaller amounts of dissociation in repeated extractions.
While both the amount and temperature of the dissociating fluid influ-
ence the degree of dissociation of the immune hemolysins, neither the
period of dissociation nor a solution of weakly alkaline reaction produce
any appreciable effect.

To obtain a more nearly pure hemolysin, the broken up erythrocytes,
lipoids and sodium chloride were removed from the sugar solution by
extraction with pure ether and dialysis and the saccharose by dialysis
and the salting out method. The immune hemolysins being insoluble in
ether and not affected by it, indicate that they are not lipoidal in nature.
The failure of the isolated immune hemolysins to dialyze through parch-
ment suggests their colloidal nature.

THE ISOLATION, PURIFICATION AND CHEMICAL NATURE OF TYPHOID ANTIBODIES

Susuma Uchida

From the Research Institute of Cutaneous Medicine, Philadelphia

In this paper is given a summary of the results of efforts to isolate, purify and ascertain the chemical nature of typhoid agglutinin and complement-fixing antibody by the same methods employed by me in a study of the immune hemolysins,[1] which were mainly those employed by Kosakai[2] in the isolation of hemolysin and of Huntoon and his associates in the isolation of pneumococcus protective antibody.[3]

General Technic.—The Rawling's strain of typhoid bacillus was employed in all of the experiments. Rabbits were immunized by intravenous injections of heat killed suspensions and their serums used when the agglutinin content was approximately 1:1000 in a macroscopic test. Antigens for the agglutination and complement fixation tests were prepared by washing 48 hour cultures from agar slants with phenolized (0.4%) distilled water or salt solution, shaking thoroughly to break up clumps, filtering through paper and heating at 60 C. for an hour.

All agglutination tests were conducted with a macroscopic method employing varying dilutions of unheated serum in amounts of 1 cc. with 1 cc. of antigen and an incubation of two hours in a water bath at 38 C. followed by over night in a refrigerator. The complement fixation tests were conducted with heated serum (60 C. for 30 minutes) in amounts of 0.5 cc. of varying dilutions with one third the anticomplementary unit of antigen and a primary incubation of 18 hours at 6 to 8 C. followed by 10 minutes in a water bath, the method being that of Kolmer's syphilis complement fixation technic. In this method normal rabbit serum in dose of 0.5 cc. of 1:20 may give nonspecific reactions, as is true of normal rabbit serum in all complement fixation tests; to avoid this possibility each serum was used in progressive dilutions of 0.5 cc. of 1:100 to 1:1000. In all agglutination and complement-fixation tests the usual controls were always included.

For sensitization 45 cc. of a heavy suspension of heat killed bacilli (25 billion per cc.) were mixed with 5 cc. of unheated immune serum (titer approximately 1:1000). After an interval of one to two hours the mixtures were centrifuged at high speed for at least an hour and the supernatant fluids removed. These were heated at 60 C. for thirty minutes and usually showed the removal of a large amount of agglutinin and complement-fixing antibody. The sediments of sensitized bacilli were then washed twice with excess amounts of sterile physiologic salt solution. Each sediment was then divided into 5 equal parts and dissociation of antibodies attempted by suspending these in the following five solutions: 10 cc. of 10% saccharose in distilled water; 10 cc. of 5.6%

[1] J. Infect. Dis., 1927, 40, p. 588.
[2] J. Immunol., 1918, 3, p. 109.
[3] Ibid., 1920, 4, p. 117.

dextrose in distilled water; 10 cc. of physiologic salt solution (0.85%); 10 cc. of 0.5% sodium bicarbonate in physiologic salt solution; and 10 cc. of distilled water. Each suspension was placed in a water bath at 60 C. for 45 minutes followed by the refrigerator over night. Each was then thoroughly centrifugated to remove all possible particles of substances, the supernatant fluids heated at 60 C. for 30 minutes and submitted to agglutination and complement-fixation tests for dissociated antibodies.

The Dissociation of Typhoid Agglutinin.—In one experiment of this kind the results of which are typical of those observed, the immune serum had a titer of 1:800. After absorption with bacilli the agglutinin titer was reduced to 1:16 showing almost complete removal of agglutinin. The first wash fluid of the sensitized bacilli contained a trace of agglutinin but the second fluid did not.

The saccharose solution of extracted sensitized bacilli gave a titer of 1:32; the dextrose solution a titer of 1:12; the sodium chloride solution 1:10 and the sodium bicarbonate solution 1:12. In other words, the saccharose yielded the best dissociation and this was found uniformly true in similar additional experiments. For example, a second immune serum gave a titer of 1:1280. After absorption the titer was 1:40. The saccharose extract of washed sensitized bacilli gave a titer of 1:20; the dextrose extract 1:2; the sodium chloride extract 1:2 and the sodium bicarbonate extract 1:5. A third immune serum gave a titer of 1:1600. The saccharose extract of washed sensitized bacilli gave 1:32; the sodium bicarbonate extract 1:4 and the distilled water 1:8.

The Dissociation of Typhoid Complement-Fixing Antibody.—Saccharose also gave best results in the dissociation of complement-fixing antibody although the tests were complicated by the fact that the saccharose extracts were slightly anticomplementary. Therefore, in summarizing results 1 shall give the highest dilution in amount of 0.5 cc. yielding strongly positive reactions with antigen and the highest dilution in the same amount yielding beginning inhibition of hemolysis without antigen (anticomplementary unit). The first immune serum gave a titer of 0.5 cc. of 1:960 and was not anticomplementary in 1:100. After absorption the serum reacted 1:20 and was not anticomplementary in 0.5 cc. undiluted. The results of tests for dissociated antibody were as follows:

Extracts	Complement Fixation	Anticomplementary
Saccharose	0.5 cc. of 1:10	0.5 cc. of 1:2
Dextrose	0.5 cc. of 1:2	Negative, 1:2
Sodium chloride	No fixation	Negative, undiluted
Sodium bicarbonate	0.5 cc. of 1:4	Negative, undiluted

The second immune serum gave a complement fixation unit of 0.5 cc. of 1:800 and was not anticomplementary in 0.5 cc. of 1:100. After absorption the serum fixation titer was 0.5 cc. of 1:40 and the anticomplementary titer 0.5 cc. of 1:4. The results of dissociation of antibody were as follows:

Extracts	Complement Fixation	Anticomplementary
Saccharose	0.5 cc. of 1:16	0.5 cc. of 1:4
Sodium bicarbonate	0.5 cc. of 1:8	0.5 cc. undiluted
Distilled water	0.5 cc. of 1:4	0.5 cc. of 1:2

In an attempt to remove the anticomplementary substances some of the extracts were passed through small sterile earthen filters and the results of one experiment with the extracts prepared of the second immune serum (given above) were as follows after filtration:

Extracts	Complement Fixation	Anticomplementary
Saccharose	0.5 cc. of 1:16	0.5 cc. undiluted
Sodium bicarbonate	0.5 cc. of 1:8	Negative
Distilled water	0.5 cc. of 1:4	Negative

In a second experiment filtration of a saccharose extract through a Mandler filter was tried with the following results, the first filtrate being passed a second time through a second Mandler:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Before filtration	1:64	1:64	1:4
After first filtration	1:32	1:16	Undiluted
After second filtration	1:16	1:8	None

It will be observed therefore, that the isolated antibodies were reduced about one half in the filters with a removal of the anticomplementary substances to about the same degree.

As observed in my experiments upon the dissociation of hemolysin, a second extraction of sensitized corpuscles usually resulted in a further dissociation of antibody. Similar results were observed in the present experiments. For example, second extractions of the bacilli used with the second immune serum gave the following results:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Saccharose	1:8	0.5 cc. of 1:8	0.5 cc. of 1:2
Dextrose	1:8	0.5 cc. of 1:2	Negative
Sodium chloride	1:4	0.5 cc. undiluted	Negative
Sodium bicarbonate	1:2	0.5 cc. of 1:4	Negative

Since the saccharose solutions appeared best for dissociation of both typhoid agglutinin and complement-fixing antibody additional experiments were conducted with them to study such subjects as optimum temperature, time and quantity for dissociation, the influence of sodium chloride, acids and alkalies and the resistance of the isolated antibodies to heat. The results are summarized as follows:

For determining the influence of temperature, saccharose solutions were employed for dissociation at 45, 55, 65 and 70 C., the technic being otherwise the same as previously described. Best results were observed at 55 and 65 C.; at 70 C. the amount of recoverable antibody (both agglutinin and complement-fixing) was somewhat less. Probably as much was dissociated but underwent destruction at this high temperature.

For determining the influence of the time allowed for dissociation, saccharose extractions of sensitized bacilli were conducted at 60 C. for 10, 20, 30, 40 and 50 minutes. The dissociation of both agglutinin and complement-fixing antibody appeared to be as good after 10 to 20 minutes as after 50 to 60 minutes; furthermore, the anticomplementary activity of the 40 to 50 minute extracts was increased probably because of the breaking up of bacilli. In general terms best results were observed by extracting at 60 C. to 65 C. for 20 to 30 minutes.

Insofar as the amount of saccharose solution in relation to the numbers of sensitized bacilli is concerned, my experiments have shown that to a slight extent the larger volumes of fluid show more antibody (both agglutinating and complement-fixing) per unit volume than the smaller volumes of extracting fluid.

Both Kosakai and Huntoon have emphasized the importance of sodium chloride in relation to the dissociation of antibodies. In my experiments equal amounts of sensitized bacilli were extracted with 10 cc. of distilled water and 10 cc. of the following solutions of chemically pure sodium chloride in distilled water: 0.25, 0.68 and 0.85%. Each suspension was kept at 60 C. for 30 minutes, centrifugated and the supernatant fluids tested for dissociated agglutination and complement fixation antibodies. The results showed more dissociation of both antibodies in distilled water than in the sodium chloride solutions; furthermore, to a slight extent, dissociation with 0.25% sodium chloride solution was better than with the 0.68 and 0.85 % solutions indicating the importance of sodium chloride in relation to antibody isolation.

The influence of hydrochloric acid and sodium hydroxide upon the process was also the subject of investigation. Hahn and Trommsdorf[4] have employed with some success N/100 sulphuric acid for the isolation of bacterial agglutinins; Liebermann and von Fennyvessy[5] report success with N/100 hydrochloric acid in saline solution while Furnhata[6] employed a solution of sodium hydroxide in sugar.

In my experiments equal numbers of sensitized typhoid bacilli were extracted at 55 C. for 45 minutes with each of the following: 10 cc. of 10% saccharose solution plus 0.5 cc. of N/10 hydrochloric acid; 10 cc. of the saccharose solution plus 0.5 cc. of N/10 sodium hydroxide; and 10 cc. of plain saccharose solution. After the usual centrifugalization the supernatant fluids were tested for dissociated antibodies with the general result that the alkalinized sugar solution showed about twice as much of both antibodies as the plain saccharose solution while the acidulated solution showed about half as much antibody content as the plain solution. It is interesting to note in passing however, that the total nitrogen content of each of the three solutions was the same, namely, 5.6 mg. per 100 cc. If this nitrogen is derived from broken up organisms it would appear that the degree of autolysis was the same in all and that the antibody content bore no relation to the total nitrogen.

    4 München. med. Wchnschr., 1900, 47, p. 413.
    5 Centralbl. f. Bakteriol., 1, O., 1908, 47, p. 274.
    6 Japan M. World, 1921, 1, p. 1.

A portion of saccharose extract was heated at 55, 60, 70 and 80 C. respectively for thirty minutes to determine the influence of heat upon the dissociated antibodies; the results of one experiment were as follows:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Unheated	1:32	1:8	1:2
55 C.................................	1:32	1:8	1:2
60 C.................................	1:16	1:8	1:2
70 C.................................	1:2	1:4	1:2
80 C.................................	None	Trace	None

These experiments have shown therefore, that the dissociated antibodies are apparently uninjured when heated at 55 C. for 30 minutes but undergo slight deterioration at 60 C., marked deterioration at 70 C. and complete destruction at 80 C., complement-fixing antibody being slightly more resistant than agglutinin.

Purification and Concentration of the Isolated Antibodies.—Since it was desirable to remove the sugar from the extraction fluids, the "salting out" method and dialysis were attempted as in the purification of dissociated antisheep hemolysin.

Saccharose solutions containing antibodies were first passed through filter candles. To 10 cc. of filtered extract were added 10 cc. of a saturated solution of ammonium sulphate. This mixture was placed at room temperature for two hours, when it became cloudy. The cloudy fluid was centrifugated. The supernatant fluid designated as "ammonium sulphate supernatant" was removed. The sediment was emulsified with 7 cc. of saline and centrifugated thoroughly and the supernatant fluid designated as "salt solution extract" was clear. The results of complement fixation and agglutination tests were as follows:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Filtered extract.......................	1:16	1:16	None
$(NH_4)_2SO_4$ supernatant	None	1:16	1:16
NaCl supernatant	1:10	1:10	None

It will be observed that the ammonium sulphate supernatant fluid (heated and unheated) was strongly anticomplimentary which was probably due to the presence of ammonium sulphate. This solution was dialyzed in order to eliminate the salt with the result that the anticomplementary activity was removed but the antibody content found almost none. It will be observed therefore, that almost all of the antibody present was precipitated by means of half saturation with ammonium sulphate so that purification by dialysis was now attempted. Nine cc. of the same filtrate which contained 6.5% sugar was placed in a parchment bag. A small amount of choloroform was added as a preservative and this was dialyzed against running tap water. After 3 dys the volume in the bag had increased to 16 cc. The sugar content of this fluid was determined by means of a saccharimeter and none was found.

During this experiment some white precipitates were formed in the dialyzed solution. The solution was centrifugated and the clear supernatant fluid was removed. It was placed in a dessicator and reduced to the original volume. The sediment was dissolved in 5 cc. of salt solution and again centrifugated. The supernatant fluid was then removed for testing. The precipitates were not all dissolved. Both supernatant fluids thus obtained were tested for typhoid antibodies with the following results:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Before dialysis	1:20	1:16	None
Dialysate (salt free).................	1:5	0	0
Isotonic dialysate	1:10	1:8	None

These attempts were repeated and the final watery solutions were used for chemical studies, but before undertaking these an attempt was made to determine what proportion of ammonium sulphate (saturated solution) gave best results as a precipitating agent, the proportions to filtered saccharose extract of sensitized bacilli being 6:14 and 8:12.

The mixtures were centrifugated after standing at room temperautre for two hours. The supernatant fluids after centrifugalization were dialyzed and concentrated to the original volume. Sodium chloride was added to render them isotonic. The sediments after centrifugating were dissolved in 8 cc. of salt solution and again centrifugated. The supernatant fluids were examined for agglutinin and alexofixagen with the following results:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Before precipitation	1:32	1:32	None
Pseudoglobulins	1:16	1:32	None
Euglobulins	1:2	1:4	None

It was found therefore, that the pseudoglobulin portion was associated with more of both antibodies than the euglobulin portion under these conditions.

Chemical Nature of Typhoid Antibodies.—Various experiments were now undertaken with these solutions of isolated antibodies to determine whether or not they were of protein nature.

Effect of Trypsin on Agglutinin and Complement-Fixing Antibody: My previous experiments with isolated antisheep hemolysin having shown that trypsin failed to digest the homolysin, a similar attempt was made to determine the effect of trypsin digestibility of typhoid antibodies.

Using 0.1% trypsin, 0.5% casein in salt and acetic acid solution I found in a preliminary test that 0.2 cc. of casein was digested by 0.5 cc. of a 0.1% trypsin. According to the nitrogen content of the extract the amount of trypsin to be employed was calculated and two tests conducted as follows: 10 cc. extract plus 0.8 cc. of 0.1% trypsin plus 1 cc. toluol; and 10 cc. extract plus 0.8 cc. salt solution plus 1 cc. toluol. These were placed at 38 C. for 18 hours and tested for antibody with the following results:

Extracts	Agglutination	Complement Fixation	Anti-complementary
Trypsinized	1:64	1:32	None
Control	1:64	1:40	None

In order to examine the activity of trypsin, acid alcohol precipitating agent was added. The control extract showed a very slight precipitate while the trypsinized extract was clearer with no precipitate. It was evident that the protein present was digested in this experiment and

that the antibodies (agglutinin and alexofixagen) after digesting of the solution with trypsin showed little difference as compared with the control.

Protein Content of Purified and Concentrated Solutions of Antibodies: Of the remaining portion of the extract containing agglutinin and alexofixagen 40 cc. were refiltered and again dialyzed in a parchment bag against running water for 50 hours. The dialysate was placed in a dessicator and the original volume obtained. This watery solution bearing antibodies was employed for chemical tests for protein with the following results: biuret, Millon's, zanthoproteic and Hopkins-Cole were negative; ninhydrin gave a trace; tungstic and phosphotungstic acid a slight opalescence; very slight with sulphosalicylic acid; doubtful with sulphur; positive with phosphorus; and the total nitrogen content was 8.4 mg. per 100 cc. while that of total nitrogen of the original serum was 1.4 gm. per 100 cc.

Another experiment was conducted in the same manner in order to determine the total nitrogen and to apply the ordinary protein reactions to the final solutions. The saccharose extract prepared in the usual manner was filtered and dialyzed, the final watery solution being placed in a dessicator to reduce it to original volume. The results of total nitrogen and protein tests were negative with acetic acid, biuret, Millon's, xanthoproteic, Heller's test and picric acid; weakly positive with sulphosalicylic acid and ninhydrin; and the the total nitrogen was 4.8 mg. per 100 cc.

It will be observed therefore, that the extracts contained some nitrogen and probably a minute trace of protein; that antibodies were present is shown by the results of agglutination (1 : 32) and complement fixation (1 : 8) tests (no anticomplementary activity).

Guinea-Pig Sensitization Tests: Experiments were now conducted to determine if the antibody solutions would produce anaphylaxis in a susceptible animal, namely, the guinea-pig.

Five cc. of a filtered and dialyzed solution containing both antibodies was injected intraperitoneally into several guinea-pigs. After two weeks incubation each guinea-pig was reinjected with 0.1 cc. of rabbit serum intravenously. These pigs showed no symptoms. On the other hand the control animals which received 0.01 cc. of rabbit antityphoid serum and then 0.1 cc. of normal rabbit serum showed definite symptoms but did not die.

This experiment has failed to demonstrate therefore, that the final solution containing both antibodies and a small amount of nitrogen produces sensitization of the guinea-pig to subsequent injection of

homologous serum (rabbit). This does not, however, definitely exclude the protein nature of the antibodies because the solution containing them might be insufficient for producing the anaphylactic reaction.

SUMMARY

In the isolation of typhoid agglutinin and complement-fixing antibody from the sensitized organisms best results were obtained with a 10% saccharose solution. Dissociation of both antibodies in saccharose solution occurred best at 60 C. for 20 to 30 minutes. The addition of sodium hydroxide to the saccharose solution increased the degree of dissociation of both agglutinin and complement-fixing antibody; the addition of hydrochloric acid reduced the degree of dissociation.

By means of a "salting out" method, dialysis and concentration of dialysate final solutions containing agglutinin and complement-fixing antibodies were obtained free of sugar and salt but containing about 4.8 to 8.4 mg. of total nitrogen per 100 cc. and a doubtful minute trace of protein.

SERUM ANTILYSINS AND METHODS FOR THEIR REMOVAL

SUSUMA UCHIDA

From the Research Institute of Cutaneous Medicine, Philadelphia

Naturally a large amount of investigation has been devoted to the subject of the nature and mechanism of the anticomplementary substances in serum in relation to complement fixation tests and especially from the standpoint of developing a practical method for their removal when found present without coincident removal or destruction of antibody. Occasionally one finds the perfectly fresh and sterile serums of some individuals anticomplementary in complement fixation tests and these, while fortunately rare, are the most puzzling of all. The majority have been observed among syphilitic individuals undergoing treatment but it cannot be stated whether or not syphilis or antisyphilitic drugs or both bear a relation to the anticomplementary state of the serum. In the great majority of instances the fresh sterile serums of human beings are not anticomplementary when tested in the amounts ordinarily employed in the Wassermann and other complement fixation tests. While Noguchi[1] found that the serums of some of the lower animals may acquire anticomplementary activity as a result of heating, yet, Kyotoku,[2] Kolmer, Rule and Trist[3] and others have not observed this to occur with human serums although, as shown by Kolmer and Trist, heating the serums of normal rabbits, dogs and mules at 55 C. for 15 to 30 minutes greatly increases their property of yielding nonspecific complement fixation reactions with various antigens of tissue extracts, bacteria, etc. In my experiments heating human serums for 30 minutes at temperatures varying from 40 to 60 C. has never rendered them anticomplementary by the unmasking of antilysins. On the contrary, heating human serums at 55 C. for 15 to 30 minutes greatly reduces anticomplementary properties and entirely removes the socalled thermolabile antilysins, so likely to develop in all serums kept for several days or longer even in a sterile condition. Indeed, the routine heating of serums at 55 C. for the Wassermann and other complement fixation

[1] J. Exper. Med., 1906, 8, p. 726.
[2] J. Immunol., 1919, 4, p. 239.
[3] Am. J. Syph., 1920, 4, p. 641.

tests is for the primary purpose of removing antilysins, the inactivation of human complement being of secondary importance.

The mechanism of the anticomplementary activity of serum remains an unsolved mystery. It is practically certain that the antilysins responsible for the phenomenon are identified with the globulin fractions of serum. This was the general conclusion arrived at by Zinsser and Johnson,[4] Kyotoku and others, although Noguchi thought that they were related more to the serum lipoids. My experiments also show that they are carried down with the globulins in the fractionation of human serum; at least this was found true of the thermostable antilysins in experiments employing hydrochloric acid as a fractionating agent in the Sach's method for their removal and likewise after fractionation with ammonium sulphate, these experiments being separately reported. Many years ago Gay[5] and Moreschi[6] sought to explain the mechanism of anticomplementary activity of serum on the basis of the occurrence of precipitation with the fixation of complement by precipitates invisible to the naked eye. This remains at present the most attractive theory although all attempts to demonstrate precipitation by means of dark field microscopy have failed in my experiments employing various mixtures of guinea-pig serum complement and strongly anticomplementary human serums. But while the mechanism of the anticomplementary activity of serums is as yet unknown, it is definitely established that bacteria and their products as well as the products of spontaneous hemolysis are capable of rendering serums anticomplementary. But whether or not such substances in plasma as excess of sugar, uric acid, creatin, creatinine, bilirubin, bile, bile salts, cholesterol, etc., may have similar effects is unknown and it was my purpose to investigate these substances in the present study. One method is to determine the anticomplementary activity of human serums showing the presence of excessive amounts of these agents; a second method is to prepare solutions of them and determine their anticomplementary activity alone and after addition to fresh, normal nonanticomplementary human serum. This is the method reported in the present paper.

The various solutions were tested before and after heating at 55 C. for 15 minutes; likewise the serum to which the solutions were added. The hemolytic system of the Kolmer complement-fixation method was employed throughout, which kept the various agents in contact with two units of complement for 18 hours at 6 to 8 C. before the addition of hemolysin and sheep corpuscles

[4] J. Exper. Med., 1911, 13, p. 31.
[5] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1905, 39, p. 603.
[6] Berlin klin. Wchnschr., 1905, 42, p. 1181.

followed by secondary incubation and the recording of results. Since some anti-complimentary agents are more active in the presence of Wassermann antigen than above, all employed in this investigation were studied in duplicate tests in which Kolmer's new antigen of cholesterolized and lecithinized alcoholic extract of beef heart was used in the usual dose of 10 antigenic units.

Each solution was tested in amounts of 0.05 to 0.4 cc. and the smallest amounts of these before and after heating at 55 C. for 15 minutes producing beginning inhibition of hemolysis (anticomplementary unit) are shown in the table.

TABLE 1

ANTICOMPLEMENTARY REACTIONS OF VARIOUS SUBSTANCES

Substances	Preparation	Anticomplementary Units				
		Substances		Substances Added to Serum		Substances Added to Serum, and Tested in the Presence of Kolmer's Antigen. Cc.
		Before Heating, Cc.	After Heating, Cc.	Before Heating, Cc.	After Heating, Cc.	
Staphylococcus albus	48 hour agar culture in salt solution	0.2	0.2	0.2	0.2	0.1
Staphylotoxin........	Mandler filtrate of a 5 days broth culture of Staphylococcus albus	0.4	(Not in 0.4)	0.2	0.4	0.1
B. subtilis............	48 hour agar culture in salt solution	0.4	0.4	0.4	0.4	0.1
Hemoglobin..........	20% solution prepared by dissolving 2 cc. of washed sheep corpuscles in 8 cc. of distilled water and rendered isotonic by adding sodium chloride	0.05	0.05	0.05	0.1	0.05
Human bile..........	20% solution	0.2	0.2	0.2	0.2	0.05
Urea.................. Creatin............... Creatinin............. Glucose.............. Ethyl alcohol........	1% salt solution...... 1% salt solution...... 1% salt solution...... 10% salt solution..... 20% in salt solution..	(Not in 0.4)	(Not in 0.4)	(Not in 0.4)	(Not in 0.4)	0.1
Bilirubin.............	0.08% in absolute alcohol containing a small amount of hydroxide	(Not in 0.4)	(Not in 0.4)	(Not in 0.4)	(Not in 0.4)	(Not in 0.4)
Uric acid.............	0.05% in distilled water plus lithium carbonate	0.1	0.1	0.2	0.2	0.1
Cholesterol..........	0.4% in 40 cc. absolute alcohol plus 60 cc. saline solution	0.2	0.2	0.2	0.2	0.1

In a duplicate set of tests equal parts of fresh, normal human serum and the various solutions were tested in amounts varying from 0.05 to 0.4 cc. before and after heating, because it is well known that serum affords some protection against the anticomplementary activity of various substances in addition to the influence of natural hemolysin tending to increase the degree of hemolysis; the serum in dose of 0.2 cc. was not anticomplementary. The tests with these solutions in equal parts of human serum were repeated after heating at 55 C. for 15 minutes in the presence of 10 units of Kolmer antigen, because the anticomplementary activity of human serums is almost always

increased in the presence of various Wassermann antigens and thereby yielding slight degrees of inhibition of hemolysis even though the serum controls show complete hemolysis.

In additional experiments the stroma of sheep corpuscles prepared according to Vedder's method [7] was found much less anticomplementary than the solution of sheep corpuscles in distilled water. Ox bile was found more anticomplementary than human bile. The bile salts, sodium glycocholate and sodium taurocholate were found to be about as anticomplementary as the bile itself.

These experiments have shown therefore, that the staphylococcus and B. subtilis, so likely to contaminate human serum, are highly anticomplementary, confirming Craig's [8] work; also that bile and its products as well as the products of hemolyzed erythrocytes furnish large amounts of thermostabile antilysins. On the other hand however, urea, creatin, creatinin, glucose and bilirubin are but feeble in anticomplimentary activity and it is entirely unlikely that these in normal or excess amounts in human serum contribute materially to anticomplementary effects; uric acid and cholesterol however, are more anticomplementary and may account for the anticomplementary activity of serum under certain pathologic conditions and especially since the anticomplementary effects of all of these are intensified in the presence of Wassermann antigen.

But the problem of the removal of thermostable antilysins from human serums submitted for the Wassermann and other complement fixation tests remains one of great practical interest and importance. From the experiments of Jaqua and Kunz,[9] Wechselman and Lange [10] and others it would appear that adsorption of anticomplementary serums with barium sulphate, kaolin, charcoal, infusorial earth, etc., may be efficacious without removing antibody at the same time but Kyotoku [2] found that while adsorption with these agents sometimes removed a portion of the thermostable antilysins, that Wassermann antibody was apt to be adsorbed in equal degree.

My experiments with blood charcoal, koalin, infusorial earth and barium sulphate, added in proportion of 0.1 gm. to 1 cc. of serum followed after an interval of one hour by centrifuging and paper filtration with heating of the supernatant serums to 55 C. for 30 minutes and testing for anticomplementary activity, have yielded similar results. I am quite sure that adsorption with these substances as well as with washed sheep corpuscles, is without practical value for the removal of thermostabile antilysins.

It is of course well known that acids (like sulphuric and hydrochloric) and alkalies (like sodium hydroxide) are highly destructive for complement. But this is not the same thing as the anticomplementary action of substances which may not actually destroy complement but bring about its nonspecific absorption or fixation. Doubtless changes in the hydrogen ion concentration of serum occur but these have not been worked out accurately in relation to anticomplementary activity. Assuming, however, that these may occur on either the

[7] J. Immunol., 1919, 4, p. 141.

[8] J. Exper. Med., 1911, 13, p. 521.

[9] Arch. internat. d. Physiol., 1909, 8, p. 227.

[10] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1909, 3, p. 528.

acid or alkaline side I have experimented extensively with the addition of varying amounts of hydrochloric acid or sodium hydroxide to anticomplementary serums to ascertain whether the thermostable antilysins could be affected, but with negative and disappointing results. It is true that the amounts of hydrochloric acid and sodium hydroxide employed were less than their hemolytic and complement destroying amounts; likewise the amounts of hydrochloric acid were less than that required for bringing about precipitation of serum proteins (globulins?), but that the use of an amount sufficient for this purpose, like 8.2 cc. of N/300 to 1 cc. of serum, as advocated by Sachs, may be a practical method for the removal of thermostable antilysins preliminary to the Wassermann or other complement-fixation tests would appear to be established; owing however, to the importance of this subject it is considered with more detail in the succeeding paper of this series.

SUMMARY

Bacteria and their products, the products of hemolyzed erythrocytes, and bile are strongly anticomplementary, while urea, creatinin, creatin, glucose and bilirubin are but feebly anticomplementary. Uric acid and cholesterol were found more anticomplementary and occurring in human serums in excessive amounts may render them anticomplementary. All were more anticomplementary in the presence of Wassermann antigen.

Adsorption of anticomplementary serums with kaolin, blood charcoal, infusorial earth, barium sulphate and corpuscles is of no practical value in relation to complement fixation tests.

Changes in the hydrogen ion concentration of anticomplementary serums were without appreciable influence upon thermostable antilysins.

REMOVAL OF ANTICOMPLEMENTARY SUBSTANCES WITH HYDROCHLORIC ACID (SACHS' METHOD)

Susuma Uchida

From the Research Institute of Cutaneous Medicine, Philadelphia

Any reliable and practical method for removing the anticomplementary properties (antilysins) from serums submitted for the complement fixation test without at the same time removing or destroying antibody, would be of much value in relation to the Wassermann test. Several years ago Sachs [1] report that the anticomplementary action of certain serums may be removed by adding hydrochloric acid to precipitate a portion of the serum globulins which appears to be responsible for the phenomenon. He emphasized that this simple procedure may be used for the serum diagnosis of syphilis as "Hilfsmittel," when the serum showed anticomplementary activity in the Wassermann reaction. The results of my investigations with this method follow:

Method.—A volume of serum (previously inactivated at 56 C. for 30 minutes) is mixed with 8.2 volumes of N/300 hydrochloric acid in distilled water. After the mixture has stood at room temperature for one-half hour, it is centrifugated and the sediment removed. To the supernatant fluid 0.8 volume of a 10% sodium chloride solution is added and used in the Wassermann test. The acid is fixed by the precipitate hence it is not necessary to neutralize the solution which represents a 1:10 dilution of original serum, that is, 1 cc. is equivalent to 0.1 cc. of whole serum. In addition I dissolved the sediment of each acidulated serum in 10 cc. of salt solution for duplicate tests and each whole serum diluted 1:10 was likewise tested as a control. Each serum therefore, was tested in three parts, namely, whole serum (heated at 55 C. for 15 minutes) diluted 1:10; the supernatant fluid of the acidulated portion; and the sediment of each dissolved in 10 cc. of salt solution. Each of these was tested with antigen for complement fixation and without antigen for anticomplementary activity, the Kolmer complement fixation method being employed throughout.

Results.—Two serums from patients under antisyphilitic treatment, which were anticomplementary in the fresh state, were available for this work. It is unusual to meet with the serums of this kind, but both of these were examined repeatedly in a perfectly fresh and sterile condition and always found strongly anticomplementary. A third syphilitic serum kept in a refrigerator for one month was found to have become anti-

[1] Berlin klin. Wchnschr., 1921, 36, p. 1075.

complementary although sterile when cultured. After heating at 55 C. for 15 minutes and testing before and after acidulation, the results with the three serums are shown in table 1.

As is well known the removal of natural hemolysins from serum by absorption with washed erythrocytes sometimes renders the serum anticomplementary. As a general rule however, the anticomplementary properties are removed by heating the serum at 55 C. for 15 to 30 minutes after removal of the sensitized corpuscles although occasionally the antilysins are thermostable. I have observed that the Sachs' method applied to serums containing thermostable antilysins after absorption of natural antisheep hemolysin, likewise results in the removal of all or a large part of the antilysins although a small part of the Wassermann antibody was removed at the same time. It will be observed therefore, that the whole serums were strongly anticomplementary; that after acidulation the supernatant fluids were free or almost free of anticomplementary substances but contained less Wassermann antibody than the original serum; and that the sediments dissolved in salt solution were anticomplementary and contained little or no antibody.

Additional serums were tested in the same manner, the results observed with 13 serums being given in table 2, as example of a series. All of these had become anticomplementary as the result of bacterial contamination and all were from patients yielding positive Kolmer reactions.

In my experience the Sachs' method has been fairly satisfactory. An accurately titrated solution of hydrochloric acid is required. Acidulation according to this technic appears to remove most of the anticomplementary substances along with a small amount of Wassermann antibody. Best results therefore, have been observed with strongly Wassermann positive serums containing small amounts of anticomplementary substances because in these the method does not remove all of the antibody while the latter are likely to be completely removed. With weakly positive Wassermann serums highly anticomplementary the results have not been very satisfactory because of the chances of removing all of the antibody during the removal of the antilysins.

Anticomplementary Substances and Serum Proteins.—Inasmuch as the globulin fractions appeared to carry the anticomplementary substances, and attempt was made to ascertain whether the antilysins are contained in the globulin fractions only or in the albumin fraction or in both fractions.

TABLE 1

Effect of Sachs' Method on Fresh Anticomplementary Serums

Amounts Dilution 1:10 Cc.	Serum 1 With Antigen Original	After Acid	Sediment	Serum 1 Without Antigen Original	After Acid	Sediment	Serum 2 With Antigen Original	After Acid	Sediment	Serum 2 Without Antigen Original	After Acid	Sediment	Serum 3 With Antigen Original	After Acid	Sediment	Serum 3 Without Antigen Original	After Acid	Sediment
1.0	4*	4	3	4	+?	3	3	3	0	1	0	0	4	4	3	4	1	3
0.5	4	4	3	3	0	+?	2	2	0	+?	0	0	4	4	2	3	0	1
0.25	4	4	1	0	0	+0	1	0	0	0	0	0	4	3	0	1	0	0
0.125	3	2	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0
0.06	1	0	0	0	0	0	0	0	0	0	0	0	+?	0	0	0	0	0
0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* 4 = ++++; 3 = +++; 2 = ++; 1 = +; 0 = negative, in all tables.

TABLE 2

Effect of Sachs' Method on Serums Made Anticomplementary by Bacteria

Serums 1:10	Whole Serums Before Treatment — With Antigen 1.0	0.5	0.25	0.125	0.06	0.03	Without Antigen 1.0	0.5	0.25	0.125	Acidulated Serums (Supernatant) — With Antigen 1.0	0.5	0.25	0.125	0.06	Without Antigen 1.0	0.5	Sediments (Redissolved) from Acidulated Serums — With Antigen 1.0	0.5	0.25	Without Antigen 1.0	0.5	0.25
1	4	3	2	0	0	0	2	1	0	0	3	2	1	0	0	0	0	4	3	0	3	0	0
2	4	3	1	0	0	0	2	0	0	0	4	2	1	0	0	1	0	2	2	0	2	2	0
3	4	4	4	0	0	0	4	4	4	0	4	0	0	0	0	2	0	4	4	0	4	1	0
4	4	4	3	0	0	0	4	4	4	0	2	1	0	0	0	1	0	2	0	0	2	0	0
5	2	2	1	0	0	0	1	0	2	0	4	3	3	3	0	0	0	3	0	0	1	0	0
6	4	4	4	0	0	0	2	1	0	0	4	3	3	0	0	1	0	2	2	0	3	0	0
7	4	4	4	0	0	0	4	3	1	0	4	3	1	0	0	0	0	3	1	0	1	0	0
8	2	1	0	0	0	0	2	0	0	0	2	0	0	0	0	1	0	3	3	0	3	0	0
9	4	4	1	3	0	0	2	0	1	0	4	2	0	0	0	0	0	2	1	0	1	0	0
10	4	4	4	2	0	0	2	1	0	0	4	3	3	2	0	1	0	3	3	0	1	0	0
11	4	4	2	1	0	0	2	1	0	0	4	1	1	4	1	0	0	3	0	0	1	0	0
12	4	4	4	3	2	0	1	0	0	0	4	4	4	4	2	1	0	4	+?	0	0	+?	0
13	3	0	0	0	0	0	2	+?	0	0	0	0	0	0	0	0	0	+?	0	0	+?	0	0

Anticomplementary serum, 2.5 cc., diluted with 25 cc. of physiologic salt solution and an equal amount of saturated solution of ammonium sulphate added. The precipitate was separated by means of repeated filtration through hardened filter paper. All of the precipitate in the filter paper was then dissolved in 25 cc. of physiologic salt solution. Both filtrate and solution were dialyzed in running water for 72 hours to remove the ammonium sulphate. The precipitated globulin was removed by centrifugation, the supernatant fluid was pipetted off, and 25 cc. of sterile distilled water added. All of the ammonium sulphate had been removed as determined with 10% barium chloride made isotonic with sodium chloride. To prevent bacterial contamination during dialyzation a drop of toluol was added. As a control a tube containing the same amount of ammonium sulphate solution and salt solution was used and dialyzed in the same manner. Wassermann tests with and without antigen were conducted with the original serum and its globulin and albumin fractions with the results shown in table 3.

TABLE 3

The Results of Wassermann and Anticomplementary Tests With Globulin and Albumin Fractions of Anticomplementary Serum

	With Antigen				Without Antigen			
Amounts in Cc.	Original Serum Diluted 1:10	Globulin Fraction	Albumin Fraction	Controls	Original Serum Diluted 1:10	Globulin Fraction	Albumin Fraction	Controls
2.0	—*	—	0	0	—	—	0	0
1.0	4	3	0	0	4	2	0	0
0.5	4	2	0	0	1	1	0	0
0.25	4	1	0	0	0	0	0	0
0.125	3	0	0	0	0	0	0	0
0.06	1	0	0	0	0	0	0	0

* — indicates no test made.

From the table it will be observed that the globulin fraction carried both the antibody and antilysins although weaker in both respects than the original serum probably because of loss of both during dialyzation and filtration. It is difficult to state just what protein fractions of syphilitic serum are precipitated by hydrochloric acid in Sachs' method, but presumably the globulins are chiefly involved. If this is true it would appear that the antilysins of serum are largely identified with the globulins. Available evidence, however, indicates that the Wassermann antibody is also carried down with the globulin fractions of syphilitic serum and if this is true it is probable that the antilysins are identified with a different fraction of the globulins than is the antibody because in Sachs' method the antilysins are largely precipitated while the antibody is but slightly removed.

CONCLUSION

The Sachs' method of testing anticomplementary serums, completely removed the thermostable antilysins from about 55% of the serums and

reduced their amounts in the remaining 45%, and also removed some of the Wassermann antibody in all of the syphilitic serums tested.

This method has been found of practical value in the Wassermann tests of slightly anticomplementary serums which contain large amounts of antibody.

INFLUENCE OF ANTICOMPLEMENTARY SUBSTANCES ON WASSERMANN ANTIBODY

Susuma Uchida

From the Research Institute of Cutaneous Medicine, Philadelphia

Serums submitted for the Wassermann test not infrequently become anticomplementary and since specimens of blood are sometimes secured with much trouble and are not easily duplicated, the question of whether or not they may be reliably tested becomes one of considerable practical importance. In efforts to evolve a method for testing such serums, the question of whether or not the complement-fixing antibody undergoes simultaneous destruction during the changes responsible for anticomplementary action, becomes one of primary importance and I have undertaken the present study to determine the fate of the Wassermann antibody in anticomplementary serums.

Anticomplementary substances (antilysins), thoroughly investigated by Kyutoku,[1] are of two kinds, namely, those contained in unheated human serums but which are completely or partially lost by heating in a water bath to 55 C. for 15 to 30 minutes and designated as thermolabile, and those not influenced by heating at this temperature and designated as thermostable. Routine heating of serums to be subjected to the Wassermann test is resorted to primarily for the purpose of removing the thermolabile anticomplementary substances and as shown by Kolmer, Rule and Trist[2] fifteen minutes at 55 C. is ordinarily sufficient. Serums older than five to seven days, however, may require heating for 30 minutes at 55 C. Fresh serums are generally free of antilysins, although an occasional anticomplementary reaction may be found, the nature of this phenomenon being unknown. Craig[3] and others have shown that bacteria growing in serum renders it highly anticomplementary. Sterile serums have been known to become anticomplementary when kept at 20 to 38 C., the rapidity of its development depending on the temperature. However, these substances are generally removed by heating to 55 C. for 15 to 30 minutes. Serums deeply tinged with hemoglobin are also apt to contain antilysins. Absorption of serums by corpuscles

[1] J. Immunol., 1919, 4, p. 239.
[2] Am. J. Syph., 1920, 4, p. 641.
[3] J. Exper. Med., 1911, 13, p. 521.

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for the purpose of removing natural hemolysin or absorption by bacteria to remove agglutinin, opsonin, etc., may render them anticomplementary, but these are generally removed by heating to 55 C. for thirty minutes, although these antilysins are sometimes thermostable. The purpose of these experiments was to determine whether the acquisition of anti-complementary properties destroyed syphilitic reagin. In other words does the Wassermann antibody in serums and spinal fluids which have become anticomplementary remain constant or undergo more deterioration than could be expected in specimens not rendered anticomplementary?

Method.—Twelve Wassermann positive syphilitic serums were selected. A portion of each was heated to 55 C. for 15 minutes and Wassermann tests made, using Kolmer's new quantitative complement fixation technic except that doses of 0.1, 0.5, 0.025, 0.0125, 0.006, 0.003, 0.0015, and 0.00075 cc. were used, for the detection of slight changes in the antibody content. At this point several procedures were adopted. Four serums were left on their clots, allowed to stand at room temperature, and were retested at intervals of a week for at least 8

TABLE 1

Wassermann and Anticomplementary Tests with Serums Kept on Clots at Room Temperature

Serum Amount Cc.	At Once		1st Week		2nd Week		3rd Week		4th Week		5th Week	
	W	A	W	A	W	A	W	A	W	A	W	A
0.1	4	0	4	2	4	4	4	4	4	4	4	4
0.05	4	0	4	0	4	2	4	3	4	4	4	4
0.025	3	0	3	0	3	0	4	2	4	2	3	2
0.0125	2	0	2	0	2	0	1	0	3	0	2	0
0.006	0	0	0	0	0	0	0	0	0	0	0	0

W = Wassermann tests; A = controls for anticomplementary activity; 4 = ++++; 3 = +++; 2 = ++; 1 = +; 0 = negative, in all tables.

weeks. Four serums were contaminated with staphylococci (in the proportion of one loopful of a 24 hour agar growth of staphylococcus albus to 5 cc. of serum) and kept at room temperature; and four sterile serums were kept in the refrigerator and subjected to a similar study. Spinal fluids, four in number were also tested in decreasing amounts of 0.5, 0.25, 0.125 cc., etc. The new Kolmer complement fixation test was used so that variations due to antigen and complement might be reduced to a minimum. Each serum was heated at 55 C. for 15 minutes and used in decreasing doses of 0.1, 0.05, 0.025 cc., etc., with antigen. These tubes showed the amount of complement absorbed or fixed by antibody and anticomplementary substances. A control was included on each dose of serum without antigen to determine the amount of complement fixed by anticomplementary substances. In this way it was hoped to ascertain the fate of the Wassermann antibody in anticomplementary serums. Spinal fluids were tested before and after heating in the same manner except that amounts of 0.5, 0.25, 0.125 cc., etc., were employed.

Results.—Table 1 shows the result with one serum which is representative of all serums left on their clots at room temperature. A gradual increase was seen in their anticomplementary properties week by week

in those which had shown no anticomplementary activity in the fresh state. Each serum gradually became tinged with hemoglobin, becoming darker and darker until at the fourth to fifth weeks, they became very sticky, putrid, and inseparable from their clots, so the tests ended with the fourth week in all but one serum. Table 2, part 1, shows the results with one serum which is representative of all serums contaminated with staphylococci. The tubes were left at room temperature throughout the eight weeks of the experiment; at the end of the first week a sediment of bacteria formed and it was necessary to agitate the tubes by means of capillary suction in order to render the serums homogeneous. Here, similar to the results shown in table 1, the anticomplementary proper-

TABLE 2

WASSERMANN AND ANTICOMPLEMENTARY TESTS

Part 1: With Serums Contaminated with Staphylococcus Albus

Amount Serum Cc.	At Once W	At Once A	1st Week W	1st Week A	2nd Week W	2nd Week A	3rd Week W	3rd Week A	4th Week W	4th Week A	5th Week W	5th Week A	6th Week W	6th Week A	7th Week W	7th Week A	8th Week W	8th Week A
0.1	4	0	4	2	4	3	4	4	4	4	4	4	4	4	4	4	4	4
0.05	4	0	4	0	4	1	4	4	4	4	4	4	4	4	4	4	4	4
0.025	4	0	4	0	4	0	4	3	4	1	4	1	3	1	4	1	4	3
0.0125	3	0	1	0	2	0	4	1	2	0	2	0	1	0	1	0	2	1
0.006	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
0.003	0	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0

Part 2: With Serums Kept in the Refrigerator

Amount Serum Cc.	At Once W	At Once A	1st Week W	1st Week A	2nd Week W	2nd Week A	3rd Week W	3rd Week A	4th Week W	4th Week A	5th Week W	5th Week A	6th Week W	6th Week A	7th Week W	7th Week A	8th Week W	8th Week A
0.1	4	0	4	0	4	0	4	3	4	3	4	3	4	4	4	4	4	4
0.05	4	0	4	0	4	0	4	2	4	1	4	1	4	1	4	1	4	1
0.025	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4	1
0.0125	4	0	4	0	4	0	4	0	3	0	4	0	4	0	3	0	3	0
0.006	3	0	3	0	3	0	3	0	2	0	2	0	2	0	2	0	2	0
0.003	1	0	1	0	1	0	2	0	?	0	?	0	0	0	0	0	1	0

ties of the serums increased while the antibody content remained fairly constant following a slight decrease the first week. Part 2 of table 2 shows the result with one serum which is representative of all serums kept in a refrigerator. The acquisition of anticomplementary activity was retarded for three to four weeks. But in this series as in the others, the Wassermann antibody remained quite uniform throughout the entire period of eight weeks of observation, although all of the serums became strongly anticomplementary during this time. The results observed with one representative spinal fluid are shown in table 3. Each was tested before and after heating at 55 C. for 15 minutes, and as expected the unheated fluids showed a higher degree of anti-complementary activity than the heated portions because of the presence of thermolabile antilysins in addition to those which were resistant to heat. The antibody content of some of the fluids underwent a slight

decrease during and following the fourth week while in others it remained fairly uniform throughout, indicating that the Wassermann antibody in cerebrospinal fluid, as in serum, is quite resistant to those substances and changes responsible for anticomplementary activity.

The general results of this investigation have shown, therefore, that the Wassermann antibody is highly resistant to the influence of those thermolabile and thermostable substances and changes responsible for the anticomplementary activity of syphilitic serums and spinal fluids. In some serums and fluids the antibody undergoes a slight decrease, as is to be expected of antibodies in general under similar conditions; certainly no increase of specific fixation of complement was noted similar to that reported by von Wedel [4] with serums from tuberculous

TABLE 3

Results of Wassermann and Anticomplementary Tests With Spinal Fluid

Amt. Fluid Cc.	At Once B W A	At Once A W A	1st Week B W A	1st Week A W A	2nd Week B W A	2nd Week A W A	3rd Week B W A	3rd Week A W A	4th Week B W A	4th Week A W A	5th Week B W A	5th Week A W A	6th Week B W A	6th Week A W A	7th Week B W A	7th Week A W A
0.5	4 0	4 0	4 4	4 1	4 4	4 1	4 4	4 1	4 4	4 1	4 4	4 1	4 4	4 1	4 4	4 2
0.25	4 0	4 0	4 3	4 0	4 0	4 0	4 0	3 0	4 3	2 0	4 3	2 0	4 4	1 0	4 4	1 0
0.125	4 0	4 0	4 0	2 0	4 0	2 0	2 0	0 0	1 0	0 0	1 0	0 0	1 0	0 0	1 0	0 0
0.06	1 0	1 0	3 0	1 0	0 0	0 0	? 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
0.03	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

B and A indicate before and after heating, respectively.

individuals. My results have confirmed those of Kolmer,[5] who found that in the serums of syphilitic individuals no increase of specific complement fixation occurred after keeping and testing the serums over a period of several weeks. Of course a serum may contain a very small amount of anticomplementary substance, too small to show an interference with hemolysis in the serum control tube, but sufficient for increasing the degree of complement fixation in the presence of antigen, but this cannot be interpreted as an actual increase or unmasking of Wassermann antibody.

CONCLUSIONS

The Wassermann antibody in the serums and spinal fluids of syphilitic persons is highly resistant to the thermolabile and thermostable antilysins developed under varying conditions.

[4] J. Immunol., 1918, 3, p. 351.
[5] Am. J. Syph., 1921, 5, p. 439.

SKIN REACTIONS WITH PNEUMOCOCCAL AND OTHER BACTERIAL FILTRATES AND EXTRACTS

Russell D. Herrold and Eugene F. Traut

From the John McCormick Institute for Infectious Diseases, and the Cook County Hospital, Chicago

Filtrates and extracts of broth cultures of pneumococci and other bacteria including streptococci, the typhoid bacillus, staphylococcus aureus of furunculosis, hemolytic and nonhemolytic colon bacilli from pyelitis, meningococci (types 1 and 2), the mucosus bacillus from chronic prostatitis, pyocyaneus and gonococci, have been tested for substances that would cause reactions in the skin. The pneumococcus and gonococcus filtrates have been studied in greater detail than the other filtrates. Herrold [1] has already reported that filtrates of broth cultures of gonococci produce positive reactions in uninfected persons (in nearly 100% of persons tested) but do not usually cause reactions in persons with active gonococcus infections. Further observations of the reactions with gonococci are to be reported. Unless otherwise specified the filtrates used in the observations now reported were obtained from 5-day dextrose phosphate broth cultures containing 0.1% dextrose and 0.1% dibasic sodium phosphate in the usual nutrient broth with the omission of sodium chloride. Most of the bacteria were grown in flat quart bottles containing 100 cc. of broth. The bottles were incubated on their flat side to get a large surface and a shallow layer of medium. However, the pneumococcus did not seem to grow profusely in the larger quantities of medium and many of the pneumococcus cultures for filtrates and extracts have been grown in ordinary test tubes with 5 cc. of broth.

Typhoid and Other Bacterial Filtrates and Extracts.—The first aim was to determine in a general way what dilutions would afford approximately a skin unit of reaction in 0.1 cc. of the filtrate. The tests were made with the filtrates of 5-day broth cultures of several pathogenic bacteria on patients of the type usually seen in the medical wards of a charity hospital. The same series of patients, however, was not available for all of the tests. The average age of the group was over 40 years. All filtrates were prepared in the same way, and the pre-

[1] J. Am. M. A., 1925, 84, p. 361.

liminary tests made with a dilution of 1 : 500 in physiologic salt solution containing 0.5% phenol. Control tests were made with the heated filtrate and the unheated broth in corresponding dilutions. The usual technic for intracutaneous tests was followed. It is evident from table 1 that a toxic substance is present in the filtrates of the gonococcus, meningococcus, typhoid bacillus, hemolytic and nonhemolytic colon bacillus (pyelitis), staphylococcus (furunculosis) and B. pyocyaneus,

TABLE 1

SKIN REACTIONS TO FILTRATES OF CULTURE OF VARIOUS ORGANISMS
5-Day Dextrose Phosphate Broth Cultures

Filtrates	Number of Patients Tested	Number of Patients with Positive Reactions*				
		Filtrate Dilutions				
		1:1500	1:1000	1:500	Undiluted	1:10†
Gonococcus (Torry 34)	6	—	—	4 (2)	—	—
Gonococcus (recent)	6	—	—	4 (1)	—	—
Meningococcus type 1	10	3 (3)	5	7 (1)	—	—
Meningococcus type 2	6	—	—	6	—	—
Typhoid bacillus	7	5	5	7	—	—
Colon bacillus, hemolytic (pyelitis)	6	5	—	6	—	—
Colon bacillus, nonhemolytic (pyelitis)	6	—	—	5	—	—
Diphtheria bacillus	6	1	4	4 (2)	—	—
Streptococcus, scarlet fever	7	1	4	4 (2)	—	—
Pneumococcus type 1	5	—	—	0	—	—
Staphylococcus aureus (furunculosis)	5	4	5	5	—	—
Pyocyaneus bacillus (Am. Type Culture Collection)	9	—	—	7	—	—
Pyocyaneus bacillus (recent isolation)	7	—	—	4 (1)	—	—
Bacillus mucosus	8	—	—	3 (1)	—	—
Control: heated gonococcus filtrate	6	—	—	0	—	—
Control: culture medium	6	—	—	0	—	—
Streptococcus						
Hemolyticus (chronic prostatitis)						
Strain 1	9	—	—	0	9	6 (2)
Strain 2	9	—	—	0	9	3 (5)
Viridans (alveolar abscess)	9	—	—	0	8	1
Viridans (chronic prostatitis)						
Strain 1	9	—	—	0	4 (3)	1
Strain 2	9	—	—	0	8	3
Strain 3	9	—	—	0	8	0 (1)

* Figures in parenthesis indicate number of persons with doubtful reactions, in addition to those with definite reactions, in tables 1 and 2; 0 indicates a negative reaction; and a dash (—), no test made.
† 15 patients tested in each instance with dilutions 1:10 of streptococcus filtrates.

even in high dilutions. B. mucosus (Friedländer's bacillus) is slightly less potent. Filtrates of cultures of green and hemolytic streptococci of prostatic origin were practically negative in the dilution 1 : 500. In further tests filtrates which produced strong reactions when diluted 1 : 500, gave similar reactions when diluted 1 : 1,000 but only a few positive results when diluted 1 : 1,500. Some of the streptococci which failed to produce reactions in dilution 1 : 500, were potent in 1 : 10 dilutions, and all undiluted filtrates were effective. Of the patients tested

with these streptococcal filtrates (undiluted and 1:10), two thirds had various types of chronic arthritis, but the reactions with these patients was no less pronounced than the reactions in the remaining one third of the patients who had other diseases.

Tests were also made with three dilutions of the more potent filtrates and with extracts from the bacterial residue obtained by centrifugation of the broth before filtration. This residue was suspended in a volume

TABLE 2

Skin Reactions with Filtrates and Extracts of Six Bacterial Varieties
5-Day Cultures in Dextrose Phosphate Broth. The Same 20 Patients
Tested with Each Preparation

Organism Preparation	Dilutions	Number of Persons		Comparison of Reactions to Filtrate (1:800) and Extract (1:10)		
		Tested	With Positive Reactions	Agreement	Relative Agreement	Opposite
Gonococcus						
Filtrate...............	800*	20	11 (2)	9	3	8
Extract...............	50	20	3 (1)			
Extract...............	5	14	11 (2)			
Meningococcus						
Filtrate...............	800	20	15 (1)	14	3	3
Extract...............	50	20	14 (2)			
Extract...............	200	11	2			
Typhoid						
Filtrate...............	800	20	16 (1)	17	2	1
Extract...............	50	20	17 (1)			
Extract...............	400	11	5 (1)			
Colon bacillus, hemolytic						
Filtrate...............	800	20	17 (2)	13	2	1
Extract...............	50	16	14 (1)			
Extract...............	400	14	9			
Streptococcus (scarlet fever)						
Filtrate...............	800	20	8 (1)	11	4	3
Extract...............	50	18	5 (3)			
Extract...............	5	16	9 (1)			
Diphtheria bacillus						
Filtrate...............	800	20	5	11	6	2
Extract...............	50	19	2 (2)			
Extract............'..	5	18	8 (1)			
Standard Dick test........	—	18	3	--	—	—
Standard Schick test......	—	20	7 (1)	—	—	—

* Filtrate dilutions 1:800 were controlled in each instance by parallel tests with dilutions 1:400 and 1:1200.

of phenolized physiologic salt solution equal to that of the removed broth. It was then placed in the icebox for several days and tested for sterility before use. The tests with both extracts and filtrates were made on the same patients, in the skin of the arms and forearms (anterior surface) and all tests on each person were completed within a few days, care being taken not to use the same area of skin twice. A few of the patients were discharged from the hospital before all of the dilutions had been tested. The results summarized in table 2 show

that the responses to extracts and filtrates differ enough to warrant further study. Apparently there is in the extracts of the bacteria a substance similar to the active substance in the filtrate but less concentrated. Further observation is necessary to determine the relative specificity of the reactions. The more accurate titration of the standard toxins for Schick and Dick tests probably accounts for the partial discrepancy in the results with the filtrates of diphtheria bacilli and scarlet fever streptococci which were included only to check the medium for its ability to produce toxins.

With the filtrate of a typhoid culture one patient with typhoid fever gave a slight positive reaction on the 23rd day of the disease, a doubtful reaction on the 29th day and no reaction on the 35th, 41st and 52nd days. Another typhoid patient had a positive reaction on the 14th day of the disease and a doubtful reaction on the 20th day. One person, a typhoid carrier for 12 years gave a doubtful reaction. One person who had been given typhoid prophylactic vaccine two years before did not give any reaction to the skin test. Eight controls with the same dilution of the filtrate of the typhoid culture gave six positive reactions, one doubtful, and in one there was no reaction.

Four patients who had received autogenous staphylococcus vaccines were tested with a staphylococcus filtrate: two had positive reactions, one negative, and one doubtful. Three patients who had received a mixture of autogenous vaccine and staphylococcus filtrate were also tested: two had negative reactions and one a positive reaction. Parker [2] obtained an exotoxin from certain strains of Staphylococcus aureus in broth. Four of seventeen strains examined produced positive reactions when tested intracutaneously in rabbits. We have obtained a similar reaction but not all of the rabbits reacted to the filtrate. A part of the rabbits tested with the other bacterial filtrates gave positive results, but usually it was necessary to inject 0.2 cc. of the undiluted filtrate.

Pneumococcal Reactions.—A type 1 pneumococcus (554, American Type Culture Collection) which was avirulent for mice in doses of 0.2 cc. of a 24 hour broth culture, and of 0.5 cc. of the filtrate, was used for all of the earlier tests (table 3).

The filtrate diluted 1:5 with physiologic salt solution was used for the intra-cutaneous tests of the first series. Injections of 0.1 cc. of the diluted filtrate, and of 0.1 cc. of control dilution of the phosphate broth were made in each instance. Although many of the subjects had parallel positive reactions to higher dilutions in the preliminary titration of the filtrate, and although the

[2] J. Exper. Med., 19, 40, p. 761.

reactions to 1:16 dilutions and in some cases to 1:32 dilutions were frequently positive it was thought safer to use a unit plus dose to obtain a great differentiation in the skin reactions of pneumonia and other patients. From the results of many tests with various filtrates it seems that a safer interpretation may be made in all skin reactions if double and triple unit doses are used in addition to the apparent single unit dose. In this way many otherwise doubtful reactions may be classified more securely as positive or negative.

In these tests about 73% of the patients with pneumonia gave no reactions to the pneumococcal filtrates, and thus differed distinctly from the other patients of whom about 85% had positive reactions. More than 75% of the positive reactions developed within 12 hours and usually the maximum reaction was reached after 18 hours, but the results were recorded uniformly at 24 hours after which time the reaction gradually subsided, although some were still pronounced after 48 hours. A small area of pigmentation may be visible for a few days after the reaction fades. A reaction is considered positive $(+)$ if

TABLE 3

Skin Reactions with a Filtrate of a Pneumococcus Type 1 Culture

5-Day Broth Culture Diluted 1:5 with Physiologic Salt Solution

Subjects	Number of Persons			
	Tested	With Positive Reactions	With Doubtful Reactions	With No Reaction
Pneumonia patients.............	38	9	4	25
Normal persons.................	49	38	5	6

larger than 1 cm. in diameter after 24 hours and doubtful if less than 1 cm. but larger than 5 mm. Absence of reaction, or a redness less than 5 mm. in diameter is classed as negative (0). The reactions to all filtrates seem to be of the same type as regards the time of appearance and of attainment of maximum size and simulate the Dick reaction rather than the Schick reaction which frequently reaches the maximum after 48 hours. Some of the 9 persons with positive reactions and of the 4 with doubtful reactions (table 3) might have been classed as negative if additional higher dilutions had been used in the test, since the serum of one of the pneumonia patients with a reaction slightly greater than 1 cm. protected a mouse from a lethal dose of pneumococci.

In repeated tests of a series of pneumonia patients (table 4) there was usually no reaction at the time of crisis, and in those patients without reactions, with a single exception, no reaction developed during the three weeks following the crisis. The reactions to filtrates of stock strains of type 1 and type 2 pneumococci, in parallel tests, were much the same, indicating that the absence of skin reactions in pneumonia patients is

not due to the use of a pneumococcus differing in type from the pneumo-coccus causing the infection.   There was agreement in fourteen of seventeen parallel tests with the two types of pneumococcus in five patients with pneumonia and twelve other patients.   The slight difference in the degree of reaction to the two filtrates was not greater than occurs with two lots of filtrate from the same organism.   Since a series of pneumonia patients who did not react to the pneumococcal filtrates, did react in nearly every instance to filtrates of typhoid and of B. coli cul-tures in suitable dilutions, the failure to react could not be considered nonspecific.   Most of the tests so far considered were made on male patients over 40 years of age.   Nine preparations from a recently isolated virulent type 2 pneumococcus (0.000,001 cc. of a 24-hour culture

TABLE 4

Skin Reactions in Repeated Tests of Pneumonia Patients with Pneumococcus
Filtrates (Avirulent Type 1)

Filtrates of 5-Day Cultures, 1:5 Dilution

Pneumonia Patients	Number of Tests on Each Patient	Results of Tests On Various Days (in parenthesis) From Onset of Diseases	
1	5	0 (14, 18, 22, 26, 46)	
2	5	0 (7, 11, 17, 23, 30)	
3	4	0 (5, 9)	+ (13, 17)
4	5	0 (5, 9, 13, 17, 35)	
5	4	0 (6, 8, 13, 17)	
6	4	0 (13, 17)	+? (6, 10)
7	4	....	+ (8, 12, 16, 20)
8	4	....	+ (8, 12, 16, 20)

lethal for mice), and a filtrate from a 5-day culture of an avirulent type 1 pneumococcus were compared in a series of 4 pneumonia patients and 4 other persons.

The type 2 pneumococcus preparations consisted of the following: a filtrate of the supernatant fluid of the centrifugated 24-hour broth culture; an extract (1) consisting of the supernatant fluid obtained by suspension of the cocci (from first centrifugation) in physiologic salt solution (volume equal to that of original culture), followed by immediate centrifugation; a second extract (2) (autolysate) of these washed cocci from the broth culture suspended in salt solution for 4 or 5 days; the supernatant broth (unfiltered) from 5-day cultures; a filtrate and two extracts of 5-day broth cultures prepared as just described for 24-hour cultures; first washings in 5 cc. physiologic salt solution, of a 24-hour pneumococcus growth on one ascites phosphate blood agar plate; 5 day icebox autolysate of the pneumococci in 5 cc. of salt solution after removal of the first fluid of the suspension in salt solution of the growth on ascites phos-phate blood agar plate.   Phenol 0.5% was added to each filtrate and extract.   A dilution of each extract and of each filtrate, 1:8 in physiologic salt solution, and 1:16 dilutions of the 1-day and of the 5-day broth filtrates were used.

The results (table 5) were consistently positive in three persons not suffering from pneumonia with the exception of the two extracts obtained by the first washings of the pneumococci from the 24-hour and 5-day broth cultures. The reactions with these two extracts at the best were doubtful in the three persons, but in later neutralization tests when 1:4 dilutions was used, fairly consistent positive reactions occurred. The four pneumonia patients failed to react with either filtrates or extracts, and so did one normal person who had had pneumonia two years before. According to the skin reactions therefore filtrates and extracts of pneumococci seem to differ very little: both generally provoke reactions in normal persons but none in pneumonia patients.

TABLE 5

Skin Reactions to Filtrates and Extracts of Virulent Type 2 Pneumococcus and to Filtrate of Avirulent Type 1

	Results of Skin Tests (Positive +, Negative 0)											
	Type 2 Filtrates				Type 2 Extracts Salt Solution					Type 2 Extracts Salt Solution 1-Day		
	5-Day		1-Day		5-Day		1-Day		Type 2 Super-natant 5-Day	Ascites 1st	Blood Agar 2nd	Type 1 Fil-trate 5-Day
Type of Case	1:8	1:16	1:8	1:16	1st	2nd (Auto-lysate)	1st	2nd (Auto-lysate)				
Pneumonia type 2.....	0	0	0	0	0	0	0	0	0	0	0	0
Pneumonia type 4.....	0	0	0	0	0	0	0	0	0	0	0	0
Pneumonia type 3.....	+	0	0	0	0	0	0	0	0	0	0	?
Pneumonia type 4.....	0	0	0	0	0	0	0	0	0	0	0	0
Pneumonia, 2 yrs. ago	?	0	0	0	0	0	0	0	0	0	0	0
Pneumonia, 8 yrs. ago	+	+	+	0	0	+	?	+	+	+	+	+
Acute bronchitis......	+	+	+	+	?	+	?	+	+	+	+	+
Acute rheumatic fever	+	+	+	+	?	?	?	+	+	+	+	+

Filtrates of 24-hour cultures seem to contain about the same quantity of toxic substance as filtrates of 5-day cultures, while the first washings of the pneumococci of either culture have distinctly less; if permitted to autolyze for five days in the icebox, however, the salt solution extracts give reactions in higher dilutions. In previous tests with the nonvirulent pneumococcus type 1 the reactions indicated that the salt solution extract (autolysate) of the residue pneumococci from the broth culture contained about one tenth as much active substance as the filtrate of the supernatant fluid (table 1). This quantitative similarity in the filtrates of virulent and nonvirulent broth cultures and the conflicting results with the extracts of pneumococcus sediments from the broth require further study.

Neutralization tests were made by mixing the serums of nonreactive pneumonia patients, with filtrates of type 1 avirulent strain and also various extracts of type 2 pneumococcus. One serum from a person

giving positive reactions was used as a control. Diluted filtrate or extract (3 parts) was mixed with serum (1 part) and left in the icebox 24 hours. The mixtures were then tested on normal persons by intracutaneous injections of 0.1 cc. Control tests with the same dilutions of the filtrate or extract in salt solution were made at the same time. The results (table 6) indicate a complete or partial neutralization in the majority of the mixtures containing the serum of convalescent pneumonia patients whose skin reactions to pneumococcal extract or filtrate were negative. Type 2 serum gave a larger number of negative reactions with the mixtures than type 3 serum. Type 4 serum was intermediate in its power of neutralization. Type 1 convalescent serum

TABLE 6

Skin Tests for Neutralization of Filtrates and Extracts of Pneumococci by Serums of Patients Convalescent from Types 2, 3 and 4 Pneumonia

Mixtures (0.6 cc. Diluted Extract and Filtrate + 0.2 cc. Undiluted Serum) Kept in Icebox 24 Hours Before Test

Pneumococcus Preparation	(12 Persons Tested, 2 with Each of 6 Serums) Number Reactive	Number Neutralized by Serum		Number Not Neutralized
		Completely	Partly	
Type 2, Virulent				
5-Day filtrate	8	5	0	3
5-Day broth, unfiltered, 1:12.........	9	2	1	6
5-Day coccus sediment salt solution extract 1, 1:3......................	10	3	2	5
5-Day coccus sediment salt solution extract 2 (autolysate) 1:6..........	6	6	0	0
1-Day solid medium salt solution extract 1, 1:12.....................	10	7	2	1
1-Day solid medium salt solution extract 2 (autolysate) 1:12.........	10	2	2	6
Type 1, Avirulent 5-day filtrate, 1:12....	10	4	3	3

was not tested. Here again the results seem to indicate that there is a similar substance in the filtrates and extracts since there is some neutralization in each and the difference is quantitative rather than qualitative.

Protective experiments were made in mice by the injection of the serums of convalescent patients with negative skin reactions and controlled by the use of serums of other patients giving positive reactions (table 7). It was found that the serums of convalescent pneumonia patients so far tested protected the mice from lethal doses of pneumococcus with the exception of one serum, and that the controls given an equal quantity of serum from patients with positive reactions usually died. The serum that did not protect, was from a patient who had developed an empyema from which a pure culture of type 2 pneumococcus was isolated. This serum did not protect against its own homologous strain or against a type 4 strain. The serums were used

in quantities from 0.2 cc. to 0.5 cc. and the controls received the same quantity of normal serum in each instance. The serums were mixed with lethal doses of 24-hour broth cultures just before injection intraperitoneally. The heart blood of all the mice that died gave pure cultures of pneumococci. Mice were given intraperitoneal injections with 0.5 cc. of the filtrate of 5-day broth culture of the virulent type 2 pneumococcus, with the immediate washings and the later icebox autolysate of the pneumococci from this broth, and also with a similar washing and autolysate of a 24-hour broth culture. All the mice had an immediate severe chill with dyspnea and prostration and appeared sick for 24 hours, but all recovered. At the end of ten days lethal doses of pneumococcus type 2 were injected. Three mice died after

TABLE 7

Protective Experiments in Mice with Serums of Pneumonia Patients and of Normal Persons

Serum	Skin Reaction of Patient to Pneumococcus Type 1 Filtrate	Type 2 Serum Number of Mice			Type 4 Serum Number of Mice		
		Tested	Protected	Not Protected	Tested	Protected	Not Protected
Pneumonia.....	0	11	9	2 (Serum of patient with empyema)	3	2	1 (Serum of patient with empyema)
Pneumonia.....	+ slightly positive	1	1	0	—	—	—
Normal.........	+	4	1	3	1	0	1
Physiologic salt solution......	—	6	0	6	1	0	1

36 hours and the heart blood gave pure cultures of pneumococci. The two mice which had previously received the pneumococcus washings and autolysate of the 5-day pneumococcus broth recovered from the injection of pneumococci.

DISCUSSION

Larson [3] mentions that approximately 50% of 17 normal persons gave positive reactions to a pneumococcus filtrate prepared by Olson [4] while three pneumonia patients who had previously received antiserum gave no reactions. He does not mention any tests in pneumonia patients who had not received antiserum. It is probable that a similar filtrate would cause no reaction in the majority of pneumonia patients even without antiserum. Further studies of pneumococcic extracts and

[3] Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 497.
[4] Ibid., p. 295.

filtrates with convalescent pneumonia serum in neutralization tests in vitro and protective experiments in mice may aid in the detection of a more specific antigen. The results of Sickles method [5] with the antiserum suggest one means of approach,—with the substitution of convalescent serums which it seems should contain the more specific antibodies formed as a result of specific stimulation by pneumococcal antigenic products during the natural course of the disease. With this known factor the more unknown antigenic and highly specific substance might be detected.

The observations made during this work also suggest the presence of a common antigenic substance in the growth products of pneumococci which may be specific, but is not necessarily confined to the specific types as determined by agglutination.

SUMMARY

It may be concluded, at least tentatively, that the gonococcus, meningococcus, typhoid bacillus, pyocyaneus bacillus, staphylococcus aureus (furunculosis) and colon bacilli of renal infections when grown in broth produce substances which act on the skin in such a manner as to merit further study. More accurate titration of the skin test unit of filtrates of streptococci from chronic prostatitis might give results of some value.

There is a distinct difference between the skin reactions of pneumonia patients and those of other persons to the filtrates and extracts of the pneumococcus. The majority of pneumonia patients give no reactions and the majority of normal persons give positive reactions. The condition preventing a reaction develops early in pneumonia and usually persists throughout convalescence.

No specific relationship appears between the skin reactions in the various pneumonia groups as classified by type agglutinating serums, and the type of pneumococcus used in the preparation of the filtrate or extract.

The results of neutralizing and protective experiments indicate that the failure of skin reaction depends on specific substances that have developed in response to the disease. Whether pneumococcal filtrates are more specific than extracts in normal salt solution will have to be determined by comparison in a larger series, but for strains which have been cultivated on artificial medium for a long time the filtrates seem more specific.

[5] J. Infect. Dis., 1927, 40, p. 369.

SPECIFIC SKIN AND TESTIS REACTIONS WITH CULTURE FILTRATES OF COCCIDIOIDES IMMITIS

Edwin F. Hirsch and Harriet Benson

From the Henry Baird Favill Laboratory of St. Luke's Hospital, Chicago

The detection of agglutinins, precipitins and complement-fixing substances in the serum of patients with coccidioidal granuloma and of animals experimentally infected with coccidioides immitis, as well as the demonstration of specific skin reactions in such hosts, has been attempted by several workers.

Cooke[1] used antigens prepared from the mycelial growth on solid culture mediums, and from the spore-like bodies present in the exudate of lesions, and concluded that, with these antigens, specific complement-fixing substances or agglutinins could not be demonstrated in the blood serum of patients, nor did these antigens cause specific skin reactions. However, precipitins were found in serum diluted 1:160 and mixed with an extract of the dried cultures. Cummins and Saunders[2] in the next year reported further studies of this kind. They could not demonstrate agglutinins, precipitins, or complement-fixing substances in the serum of patients and animals with the disease. They report that the concentrated filtrate from heated and unheated broth cultures caused a cutaneous reaction in some of their infected rabbits, and to a less degree in normal rabbits. Davis[3] could not detect agglutinins in the serum of a patient he studied, but obtained complement fixation reactions with a concentrated culture as antigen. An intradermal skin reaction occurred with killed homologous organisms decidedly more intense than that following the injection of similar material prepared from cultures of sporothrix, blastomyces, or of agar.

Studies by the authors mentioned of a specific skin reaction in patients or animals infected with coccidioides immitis have not established concordant results. Histologically, the lesions produced by coccidioides immitis closely resemble those caused by B. tuberculosis,[4] and they differ markedly from a foreign body reaction such as occurs in tissues surrounding relatively inert particles. It seems likely, therefore, that some soluble substance is produced by coccidioides immitis which resembles in some respects, at least, the tuberculin produced by the

[1] Arch. Int. Med., 1915, 15, p. 479.
[2] J. Med. Research, 1916, 35, p. 243.
[3] Arch. Dermatol. and Syphilol., 1924, 9, p. 577.
[4] Hektoen, L.: J. Am. M. A., 1907, 49, p. 1071.

tubercle bacillus, and that skin reactions can be obtained in patients
and animals infected with coccidioides immitis which are like those with
tuberculin in tuberculous hosts. Further studies of skin reactions were
made on a negress with light colored skin, who was afflicted with
coccidioidal granuloma. Concise résumés of the disease have been
reported from time to time, recently by Bump.[5]

According to statements by foster parents of the patient, the negress, 9
years of age, in June, 1925, returned to Chicago from Phoenix, Arizona, where
she had visited for six months. For one and one-half years before her stay
in Arizona she had been in Los Angeles, California. She was admitted to
St. Luke's Hospital, Chicago, Jan. 21, 1926, to the service of Dr. S. C. Plummer,
because of a painful swelling of the base of the left thumb and of the left knee
that had been noticed about three weeks. The tissues of the thumb were tense
and fluctuated as though containing an exudate. On January 22, 1926, this
abscess was aspirated and a blood-stained, yellow, turbid, and viscid exudate
was obtained. A mold was isolated in cultures of the exudate, and in moist
cover glass and slide preparations mixed with 10% sodium hydroxide, oval
double-contoured spherical structures, some containing endospores, were found.
The mold was further studied by inoculations in guinea-pigs and was identified
as coccidioides immitis. On March 24, 1926, the left knee was aspirated and by
cultures and in moist preparations coccidioides immitis organisms were found.
In liquid cultures, the mold does not ferment 0.5% concentrations of dextrose,
lactose, saccharose, mannitol, salicin, raffinose, xylose, arabinose, dextrin, levu-
lose, inulin, or maltose. It is highly proteolytic, gelatin (14%) being liquified in
two days, and milk coagulated and peptonized in three days. The x-ray exam-
ination (by Dr. E. L. Jenkinson) demonstrated a destruction of the distal third
of the first metacarpal bone of the left hand, and of the anterior, inferior half
of the left patella. Both lungs were studded with small, opaque masses. The
patient at the time of this report is physically in as good condition as when
admitted; her daily temperature is normal, and the infection is making little if
any progress.

Coccidioides immitis grows abundantly in the usual simple culture
mediums. For the intradermal skin tests the mold was inoculated into
peptone broth prepared with placenta meat extract to which were added
0.1% dextrose and 0.2% Na_2HPO_4. After varying intervals of growth
the liquid medium was filtered (Berkefeld N filter) and the filtrate
tested for sterility. F_1 was prepared after 18 days' growth, F_2 after
25 days, F_3 after 40 days. Another filtrate (F_4) was prepared from a
culture in synthetic medium (0.5% NaCl, 6% ammonium lactate, and
0.2% dibasic potassium phosphate)[6] after 28 days' growth.

Four guinea-pigs inoculated 4/12/26 subcutaneously in the right
groin with exudate from the knee and three normal pigs, were tested on

[5] J. Infect. Dis., 1925, 36, p. 561.
[6] A modification of the medium suggested by Braun and Cahn-Bronner: Centralbl. f.
Bakteriol., 1, O., 1921, 86, p. 1.

the 7th day (4/19) by injecting intracutaneously 0.1 to 0.2 cc. of F_1.
The reaction in all of the infected animals was about the same at the end
of 24 hours, there being a moderate reddening and swelling of the skin
injected with the filtrate. There was no reaction in the skin of the con-
trol animals.

On 4/26 (14th day) the four infected guinea-pigs, one other inoc-
ulated the same date, and three control guinea-pigs were tested with F_2
in the way indicated. In the infected animals after 24 hours there was
a markedly reddened wheal 18 to 22 mm. in diameter with some blanch-
ing of the center; after 48 hours this was a reddened, swollen region
15 mm. in diameter. The reaction in the control animals after 24 hours
was simply a slight reddening 3 to 10 mm. in diameter, and this after
48 hours had disappeared.

Similar skin tests with F_2 were made the same day (4/26) on the
patient and a human control. The effect of the broth filtrate injection
was compared in both by another injection of the culture medium only.
At the end of 24 hours the skin reaction of the patient consisted of a
swollen red place 85 x 40 mm., in the center of which was a wheal 15 mm.
in diameter. The place injected with the medium only was reddened
slightly, less than 1 cm. in diameter. In the human control the F_2 injec-
tion caused a slight redness about 20 mm. in diameter which had disap-
peared almost completely at the end of 48 hours. The skin reaction in
the patient after 48 hours was a reddened region 70 x 40 mm. Another
skin test in the patient with F_2 caused the same reaction, and further
tests with F_3 were similar. Broth culture filtrates of two other strains
of coccidioides immitis obtained from Dr. F. Proescher, San Jose,
California, caused similar immediate and delayed skin reactions in the
patient.

The effect of heat on the activity of F_2 was tested by heating for 30
minutes one portion at 60 C. and another portion at 80 C. The skin
tests were made as described in the five infected guinea-pigs and in three
healthy animals. After 24 hours in the infected guinea-pigs there were
edematous wheals with some blanching 15 to 20 mm. in diameter. There
was no difference between the reactions caused by the filtrate heated to
60 C. and that heated to 80 C. A slight reddening of the skin 5 mm.
in diameter occurred in the normal guinea-pigs. The same solutions
were used in skin tests on the patient and on the human control. No
reactions occurred in the healthy person, but in the patient after 24 hours
there were red and swollen places 40 x 25 mm. Skin tests in other

infected guinea-pigs and rabbits with these filtrates (F_1, F_2, F_3) were like the ones mentioned.

Although the growth of coccidioides immitis in the synthetic medium is less abundant and slower than in the dextrose broth, the filtrate (F_4) obtained caused skin reactions in the infected animals and in the patient of the same nature as those mentioned, although only about two-thirds as strong as, for example, those produced with F_3.

Some mention should be made of the immediate skin reaction which occurs in the patient following the injection of 0.1 cc. filtrate. Within a few minutes there is a wheal 15 to 20 mm. in diameter with peripheral radiations like the skin reaction of a patient with hay-fever following the application of a specific pollen extract. This subsides considerably within an hour, only a slight swelling being noted, and about 6 to 12 hours later the redness and further swelling appear, reaching their maximum in 24 to 36 hours. With some of the tests in guinea-pigs and rabbits, small regions of necrosis appeared in the center of the reddened skin. When the redness has disappeared the skin surface may be rough with desquamating epidermis.

After five days electrodialysis of 30 cc. of F_4 slight flocculation occurred. The floccules were centrigated from the supernatant liquid and dissolved in distilled water containing a few drops of N/100 NaOH. The clear liquid was neutralized with one drop of N/10 HCl and used with the electrodialyzed supernatant liquid for skin tests. Skin reactions like those mentioned occurred in guinea-pigs infected with coccidioides immitis, both with the solution of floccules and with the supernatant liquid. These solutions caused no reaction in a healthy animal.

When the testis of a guinea-pig infected with coccidioides immitis is injected with 0.1 to 0.15 cc. of a liquid medium filtrate like the ones mentioned, there occurs a marked reddening and swelling of the gland within 24 to 36 hours, a reaction much greater than that following such an injection into the testis of a normal guinea-pig. After the immediate reaction has subsided, the gland diminishes in size. A guinea-pig infected with coccidioides immitis May 12, 1926, received June 21, in the right testis, 0.15 cc. of F_3. After 25 hours he was killed. The right testis markedly reddened, swollen and tense was 22 by 13 by 15 mm. and the serous surface is dull. The left testis was 12 by 8 by 7 mm. The testis of a normal guinea-pig injected at the same time was practically unaltered, the right testis being 17 by 12 by 11 mm., the left 17 by 11 by 11. Another guinea-pig infected May 12, 1926, and given an injection June 21 in the right testis with F_3 in the same way was killed

June 30, 1926. The right testis was 20 x 10 x 6 mm., the left was 22 x 14 x 12 mm. These gross reactions resemble those caused in the testis of tuberculous guinea-pigs by the injection of tuberculin.[7] Other experiments of this kind were similar.

SUMMARY

The results obtained indicate that the growth of coccidioides immitis in liquid mediums liberates a soluble specific substance which in animals infected with coccidioides immitis and in patients with coccidioidal granuloma causes skin reactions like those occurring with tuberculin in tuberculous animals. The immediate skin reaction resembles the wheal produced by the specific pollen extract in a patient with hay-fever. This soluble specific substance is not destroyed by heating to 80 C. for 30 minutes. The reactions with the filtrate of a synthetic medium culture are like those obtained with filtrates of ordinary broth cultures. Prolonged electrodialysis of the synthetic medium filtrate causes the separation of white floccules. These dissolve readily in distilled water containing a few drops of N/100 sodium hydroxide and the solutions cause skin reactions like those of the original filtrate. Not all of the specific substance is flocculated by electrodialysis.

The intratesticular injection of liquid culture filtrates causes a gross reaction in guinea-pigs with coccidioidal granuloma like that in the testes of tuberculous animals after the injection of tuberculin.

[7] Long: Am. Rev. Tuberc., 1924, 9, p. 215. Long and Seyfarth: Ibid., p. 254.

THE SPECIFIC SUBSTANCE OF COCCIDIOIDES IMMITIS

Edwin F. Hirsch and Dorothy D'Andrea

From the Henry Baird Favill Laboratory of St. Luke's Hospital, Chicago

Filtrates of broth and liquid synthetic culture mediums in which coccidioides immitis has grown for some time have been found[1] to cause specific skin reactions in animals experimentally infected and in a patient with coccidioidal granuloma. Because of the relatively simple nature of the synthetic medium in which the mold grows, it seems possible to isolate from such filtrates the substance giving the specific skin reaction and to learn some facts regarding its nature. The medium used is a modification of that suggested by Braun and Cahn-Bronner.[2] It contains distilled water with 6% ammonium lactate, 0.2% K_2HPO_4, and 0.5% NaCl. After five months growth at incubator temperature the mold has formed a thick mycelium throughout the culture medium. The mycelium is removed from the liquid portion by centrifugation and filtration. To one volume of the clear liquid two volumes of 95% ethyl alcohol are added. The mixture becomes turbid and after standing at room temperature, fine white floccules separate. These are removed by centrifugation, washed with 95% ethyl alcohol and redissolved in a small volume of distilled water. Two volumes of 95% ethyl alcohol are added, usually without causing more than a faint turbidity. Two or three drops of a saturated sodium chloride solution added to a 15 cc. volume cause a marked turbidity and subsequent flocculation. The precipitate is washed with 95% ethyl alcohol, and is again dissolved in distilled water and reprecipitated with alcohol. These floccules are washed with 95% alcohol, absolute alcohol, and then ether. The white powder is dried in a dessicator. No precipitates of this kind were obtained from uninoculated mediums.

It dissolves readily in water, 0.9% NaCl solution, N/10 NaOH and N/10 HCl. Because of the relatively small amounts of material recovered, extensive analyses have not been possible. Quantitative analyses for protein nitrogen and for carbohydrate after hydrolysis have

[1] Hirsch. E. F., and Benson, H.: J. Infect. Dis., 1927, 40, p. 629.
[2] Centralbl. f. Bakteriol., 1, O., 1921, 86, p. 1.

given the results contained in the table. The nitrogen estimations were made according to the Folin and Wu[3] microtechnic with 1 to 2 mg. quantities dissolved in N/1 NaOH and aerated for two hours to guard against ammonia nitrogen. The carbohydrate estimations were made according to the Folin and Wu[4] technic for blood sugar determinations with 1 to 2 mg. samples hydrolyzed six hours in N/2 HCl. Other quantities were used in making skin tests.

Having isolated from synthetic culture filtrates a nitrogen and carbohydrate containing substance which in high dilutions gives specific skin reactions, the method was applied to plain broth and dextrose broth culture filtrates. These cultures had grown six and one-half months. The results were the same (table), the yield of precipitated material of course, being considerably greater than with the synthetic medium filtrates.

Culture Filtrates	N, %	Reducing Sugar After Hydrolysis (Measured as Dextrose) %	Skin Tests With 0.05 to 0.1 Cc. of Solution Containing Mg. per Cc.	Reaction
Synthetic medium.................	3.0	21.8	0.000,05 0.000,1	Slight +
Dextrose broth....................	3.2	20.8	0.001*	+
Dextrose broth....................	3.0	35	0.000,05 0.000,1	Slight +
Dextrose broth....................	4.4	46.5	0.000,05	+
Nutrient broth....................	3.6	39.6	—	—
Nutrient broth....................	3.1	31.0	0.000,05	+
Nutrient broth....................	4.1	39.4	0.000,05	+

* Higher dilutions not tested.

Unhydrolyzed solutions contain no reducing sugar, but react strongly with the Molisch test. The phenylhydrazine test for the formation of osazones was made with a hydrolyzed solution of material obtained from broth filtrates. Crystalline structures resembling closely the dextro-sazone were found in microscopic preparations of the liquid. The amount of material available, however, was too small for melting point or other determinations.

The skin tests were made with the patient, and with healthy adults by intracutaneous injections of 0.05 to 0.1 cc. of the different solutions. In the patient these injections cause skin reactions of two kinds, an

[3] J. Biol. Chem., 1919, 38, p. 81.
[4] Ibid., 1920, 41, p. 367.

immediate, occurring within fifteen minutes, and another visible at its maximum usually after twenty-four hours. The immediate reaction is the more marked and consists of an edematous wheal starting with a bleb 2 to 4 mm. in diameter caused by the injected fluid and attaining within ten or fifteen minutes a diameter of 10 to 12 mm. in appearance much like an urticarial wheal. Along the periphery frequently there are slender prolongations. About the central wheal is a reddening of the skin 15 to 25 mm. wide. The later reaction is a reddening of the skin 10 to 15 mm. in diameter. This reaction is much less marked with solutions of the flocculated material than has been noted with the whole filtrates. Perhaps this difference is due to the variations in the amount of actual specific material injected. No such reactions occurred with the control injections. Alcohol precipitation does not flocculate all of the specific substance from the filtrates.

DISCUSSION

A recent report by Heidelberger and Goebel [5] gives further results of investigations that have been made in determining the nature of the specific polysaccharide of type 3 pneumococci. Mention is made in this report of results already published regarding the specific polysaccharide isolated from culture filtrates of types 1 and 2 pneumococci, and of similar carbohydrate containing preparations with specific properties obtained from the Friedlander bacillus, the tubercle bacillus, the yeast, and the streptococcus viridans. With these are included also the "residual" antigens of Zinsser and Parker. The purified preparations of Heidelberger and Goebel are without nitrogen or ash constituting substances. Immunization, however, has not been accomplished with these carbohydrate preparations, although the specific reaction (flocculation) is still maintained. It seems that the preparations obtained from culture filtrates of the mold, coccidioides immitis, belong to this class of substance, although nitrogen-free preparations, as yet, have not been obtained. No experiments have been made for determining the ability of these preparations to immunize animals.

The stimulation of skin reactions in the patient by highly dilute solutions of the coccidioides immitis preparations is comparable in activity with the flocculation observed with similar solutions of the purified specific substances of pneumococci and other bacteria when mixed with homologous immune serum.

[5] J. Biol. Chem., 1926, 70, p. 613.

SUMMARY

From a synthetic protein-free culture filtrate of coccidioides immitis a specific substance containing nitrogen and carbohydrate is recovered, which in high dilution causes skin reactions in a patient with coccidioidal granuloma. Similar preparations have been recovered from broth culture filtrates. The dried specific substance is a white powder readily soluble in water, 0.9% sodium chloride solution, dilute alkalies and acids, contains about 3 to 4 per cent nitrogen and, on hydrolysis, 20 to 40% reducing sugar measured as dextrose. The osazone prepared from a hydrolyzed solution resembles in structure the dextrosazone.

SENSITIZATION OF GUINEA-PIGS WITH BROTH CULTURE FILTRATES AND WITH THE KILLED MYCELIUM OF COCCIDIOIDES IMMITIS

Edwin F. Hirsch and Dorothy D'Andrea

From the Henry Baird Favill Laboratory of St. Luke's Hospital, Chicago

Skin tests with filtrates of old liquid cultures of coccidioides immitis in animals infected experimentally and in a patient chronically sick with the disease have demonstrated reactions of two kinds, an immediate, resembling an urticarial wheal, and a delayed, present at its maximum as a marked reddening and swelling of the skin tissues after twenty-four hours. These reactions indicate specific sensitization and permit the conclusion that the growth of the organism in tissues or cultures liberates a soluble specific substance whose presence in tissues may be considered to play such part in the formation of lesions as do the products of the tubercle bacillus in tuberculous lesions and whose presence in cultures is analogous to tuberculin. Indeed, the lesions produced by coccidioides immitis resemble very closely those caused by the tubercle bacillus. Recently Zinsser and Tamiya [1] have reported an experimental analysis of bacterial allergy. They mention as manifestations of bacterial allergy the immediate urticarial skin reaction, and the delayed skin reaction. The reaction noted in infected animals with intracutaneous injections of coccidioides immitis filtrates are like these. Among the experiments reported by Zinsser and Tamiya are some made with extracts of the tubercle bacillus. They were able to sensitize guinea-pigs to give skin reactions with tuberculin, O. T., by injections with the nucleoprotein of the tubercle bacillus, and even by injections of O. T. According to their statements sensitization of guinea-pigs by injections of O. T. had not been observed by other investigators or by themselves previously. With relatively large amounts of O. T. in proper dilutions, however, sensitization occurs like that with the nucleoprotein substance of the bacillus.

At the time these results were published experiments with guinea-pigs had been completed in which sensitization with a filtrate of an old broth culture of coccidioides immitis had been accomplished. The filtrate used was prepared from a broth culture of the organism isolated

from a patient and was grown at incubator temperature for six months. Five guinea-pigs were given subcutaneous injections with 1 cc. of the filtrate on 11/1, 11/2, 11/4, 11/5, 11/6, 11/8 and 11/9, and five others received injections on the same dates with 1 cc. of broth medium. Skin tests were made on all guinea-pigs 11/3 with a coccidioides immitis filtrate that produced a strong skin reaction in the patient. No immediate or delayed reactions occurred, nor with injections of the broth medium only. Similar tests were made 11/11 without causing reactions. These when repeated 11/15 resulted in moderate delayed reactions in the guinea-pigs given injections of filtrate and no reactions in the animals treated with broth. Tests were made 11/19 with a solution of the specific substance [2] prepared from a plain broth culture filtrate and containing 0.5 mg. per cc. of 0.9% sodium chloride solution. Moderate to slight delayed skin reactions occurred in the guinea-pigs treated with filtrate but none in the control animals.

Because of these suggestive results and the generally known sensitization of guinea-pigs toward tuberculin by the injection of killed tubercle bacilli, other experiments were made in which guinea-pigs received intraperitoneally small amounts of the killed coccidioides immitis mycelium reduced to a dry powder, and suspended in 0.9% sodium chloride solution. The mycelium from old cultures was removed by centrifugation, treated successively with 95% alcohol, absolute alcohol, and ether, and was then dried thoroughly in a dessicator and reduced to a light brown powder in a mortar. Three guinea-pigs were injected intraperitoneally 11/20, 11/26, and 11/29 with 0.5 mg. of the dried powder prepared from the mycelium of a broth culture grown for seven months in the incubator. Skin tests with an active culture filtrate and with broth medium as control were made 12/6 in these and in normal animals. Immediate reactions and delayed reactions in the form of reddened wheals from 10 by 10 mm. to 25 by 30 mm. occurred in the mycelium injected guinea-pigs. No reactions occurred in the control animals or with the broth medium injections. Broth culture filtrates of two other strains of coccidioides immitis obtained from Dr. F. Proescher, San Jose, California, also caused skin reactions, but these were less intense probably because the filtrates used were of cultures only 17 days old. A solution of the soluble specific substance (0.5 mg. per cc. of 0.9% salt solution) prepared from one of these filtrates also caused a marked delayed skin reaction. The nodules in the omentum and on the peritoneal surfaces found after the animals were killed

[2] Hirsch, E. F., and D'Andrea, D.: J. Infect. Dis. In print.

contain histologically chronic granulation tissue resembling that caused by B. tuberculosis in which are large masses of polynuclear leukocytes densely arranged around aggregates of the injected granular material. No coccidioides immitis organisms are present.

Two other guinea-pigs received intraperitoneally 0.5 mg. of the mycelium 12/1, 12/4 and 12/9. Intracutaneous tests in these as well as in a normal guinea-pig were made 12/20 with a filtrate from an old culture; with a solution containing 0.5 mg. of the specific substance (prepared from a culture of the same strain) per cc. of 0.9% sodium chloride; with a filtrate of a culture from Dr. Proescher; and also with a broth medium control. All the solutions containing coccidioides immitis culture filtrate material caused moderate to marked skin reactions, especially the filtrate from the old culture. The control injections caused no reactions.

CONCLUSIONS

It is possible, according to the results of these experiments, to sensitize guinea-pigs with broth culture filtrates of coccidioides immitis and with the killed, dried mycelium of the mold. In such sensitized animals immediate and delayed skin reactions are produced by filtrates of the same strain and of other strains of the mold, as well as with solutions of the soluble specific substance obtained from broth culture filtrates. These results are-like those obtained by sensitizing guinea-pigs to tuberculin, O. T., with injections of either O. T. or killed tubercle bacilli.

SPECIFICNESS IN THE PRECIPITIN REACTION OF THYROGLOBULIN

LUDVIG HEKTOEN, HERBERT FOX, KAMIL SCHULHOF

From the John McCormick Institute for Infectious Diseases, Chicago, and the Laboratory of Comparative Pathology of the Zoological Society of Philadelphia

The results of studies with the precipitin reaction of thyroglobulin, especially human and canine, have been reported in several papers.[1] It has been pointed out that this reaction, while specific for thyroglobulin, is not limited to the homologous antigen, that is, it is not species-specific. We now wish to record the results of observations on the range of action of precipitins for thyroglobulins from wild animals that died in captivity as well as from domesticated species.

The thyroglobulin was prepared following Oswald's method as now described:

The thyroid is freed from fat and connective tissue and torn into small pieces, which are washed in water until almost white, and then extracted in the icebox for two days with 0.85% salt solution containing a little thymol. The extract is centrifugated thoroughly, the fluid mixed with half its volume of saturated solution of ammonium sulphate and then centrifugated again. In favorable material only a little precipitate forms at this time, but most of this, except for cells, consists of thyroglobulin, which consequently is lost. The clear supernatant fluid is now mixed with ⅓ of its volume of saturated solution of ammonium sulphate, and this brings the concentration of the mixture to ½ of a concentrated solution of ammonium sulphate. The resulting precipitate is washed with a half concentrated ammonium sulphate solution, dissolved in salt solution and reprecipitated once or twice by adding an equal amount of saturated solution of ammonium sulphate. If it is desired, the ammonium sulphate in the final solution may be removed by dialysis. For precipitin tests the final solution should have a concentration of 0.85% sodium chloride. It can not be emphasized too strongly that, in order to obtain a good result, considerable thyroid material must be wasted. It is especially important to discard parts that remain pink after the preliminary washing in water.

The thyroglobulin (and other eventual protein) content of the final solution may be estimated by comparing the turbidity on adding an equal amount of a

[1] Hektoen, Ludvig; and Schulhof, Kamil: The Precipitin Reaction of Thyroglobulin, J. Am. M. A., 1923, 80, p. 386. Hektoen, Ludvig; Carlson, Anton J., and Schulhof, Kamil: The Precipitin Reaction of Thyroglobulin, Presence of Thyroglobulin in the Thyroid Lymph of Goitrous Dog, J. Am. M. A., 1923, 81, p. 86. Carlson, Anton J.; Hektoen, Ludvig; and Schulhof, Kamil: Experimental Increase in the Rate of Output of Thyroglobulin by the Thyroid Gland, Am J. Physiol., 1925, 71, p. 548. Hektoen, Ludvig; Kanai, Paul H., and Dragstedt, Lester R.: A Study of Protein Absorption from the Digestive Tract by the Precipitin Tests, with Especial Reference to Thyroglobulin, J. Am. M. A., 1925, 84, p. 114. Hektoen, Ludvig; and Schulhof, Kamil: The Precipitin Reaction of Thyroglobulin. Specificness; Presence of Thyroglobulin in Human Thyroid Veins; Production by Rabbit of Precipitin for Rabbit Thyroglobulin; Thyroglobulin in the Foetal Thyroid and in Exophthalmic Goiter, Proc. Nat'l. Acad. Sc., 1925, 11, p. 481.

20% solution of sulphosalicylic acid with the turbidity produced under the same conditions in a protein solution of known strength. For preliminary rough estimates one may use as a standard normal human serum one part and salt solution six parts; this gives a protein solution 1 to 100 from which dilutions can be made as required. The solutions of thyroglobulin we have used, varied in estimated strength from 1:100 up to 1:3200.

Rabbits are immunized by intravenous injections of increasing quantities of thyroglobulin, usually 2, 4, 8, 12 and 15 cc. of solution, no matter the actual strength, at intervals of three days. Commonly the serum is found to be rich in precipitin on the fourth or fifth day after the fifth injection. In a few cases three daily injections of increasing amounts, repeated if necessary after an interval of a few days, have given good results. The precipitin tests are made by the contact or layer method and the results read after one hour at room temperature. In all cases tests are made with increasing dilutions of thyroglobulin solutions of known strength (at least approximately) in order to go well beyond the range of any possible so-called prozone in the stronger concentrations and also to determine in a general way the precipitin titers of the antiserums. In this paper the results are recorded as positive ($+$) or negative (0).

With care it is possible by the method we have used to prepare thyroglobulin so that it is practically free from blood proteins as determined by tests with the proper antiserums. We have obtained precipitin serums for diverse thyroglobulins that did not give any reaction with the corresponding blood serum. In other cases the antithyroglobulin serum reacted with low dilutions of the blood serum, but in such cases only with the homologous. We have had precipitin serums for human, dog and beef thyroglobulins that gave no reaction with the respective blood serum or blood proteins (fibrinogen, euglobulin, pseudoglobulin, albumin, hemoglobin). While human thyroglobulin removed all the precipitin from the corresponding antiserum, human serum did not seem to have any effect whatever. The one antiserum for chicken thyroglobulin studied up to this time had a high titer, but reacted also with chicken serum and chicken fibrinogen. The precipitins for serum and fibrinogen, however, were removed completely by absorption with either serum or fibrinogen, and without serious reduction in the precipitin for thyroglobulin. In this case, then, the thyroglobulin contained fibrinogen and possibly other blood proteins.

Table 1 shows, first, that the chicken thyroglobulin stands out as strictly specific. Its precipitin serum does not react with any of the mammalian thyroglobulins, and their antiserums do not react with the

chicken thyroglobulin. Clearly it would be of interest to study fowl thyroglobulins on a considerable scale, with reference to their relations to each other as well as to mammalian thyroglobulins.

In the next place the tabulation reveals that the various precipitin serums against mammalian thyroglobulins each reacted with other thyroglobulins than the one used in immunizing. In fact, the serum against bear thyroglobulin reacted with all the mammalian thyroglobulins

TABLE 1

ABSENCE OF SPECIES SPECIFICNESS IN PRECIPITIN REACTIONS OF THYROGLOBULIN

Thyroglobulins	Serum of Rabbits Injected with Thyroglobulin from								Normal Rabbit Serum
	Bear	Beef	Chicken	Dog	Horse	Man	Sheep	Zebra	
Anteater (Myrmecophaga tridactyla)...	+	+	0	+	+	+	+	0	0
Baboon (Papio porcarius)...............	+	+	0	+	0	+	+	+	0
Baboon (Papio maimon).................	+	0	0	+	0	+	+	..	0
Bear, Grizzly (Ursus horribilis)..........	+	0	0	+	+	0	+	+	0
Bear, Brown (Ursus beringiana).........	+	0	0	+	0	+	+	+	0
Bear, Polar (Ursus maritimus)..........	+	0	0	+	..	..	+	+	0
Beef..	+	+	0	+	+	+	+	+	0
Cat, Fishing (Felis viverrina)............	+	0	0	0	0	0	0	..	0
Chicken (Gallus domesticus)............	0	0	+	0	0	0	0	0	0
Coati (Nasua nasua).....................	+	0	0	+	0	0	+	0	0
Deer (Mazama virginiana)...............	+	0	0	+	+	0	+	+	0
Deer (Cervus elaphus)....................	+	+	0	+	0	0	+	+	0
Deer (Cervus axis).......................	+	+	0	+	+	0	+	+	0
Deer (Cervus dama)......................	+	+	0	+	0	0	+	+	0
Deer (Cervus sika).......................	+	+	0	+	..	..	+	+	0
Dog..	+	0	0	+	0	+	+	+	0
Fox (Vulpes pennsylvanicus)............	+	+	0	+	..	..	+	+	0
Hog..	+	0	0	+	+	+	+	0	0
Horse......................................	+	0+	0	+	+	+	+	+	0
Jackal (Canis aureus).....................	+	0	0	+	0	0	+	..	0
Kangaroo (Macropus robustus).........	+	0	0	+	0	0	0	0	0
Man..	+	0	0	+	+	+	+	+	0
Monkey (Cercopithecus nictitans).......	+	0	0	+	0	0	+	+	0
Porcupine (Hystrix cristata)............	+	0	0	+	0	0	+	..	0
Rabbit.....................................	+	0	0	0	0	0	0	+	0
Raccoon (Procyon cancrivorus).........	+	0	0	+	+	+	+	+	0
Rat..	+	0	0	0	+	+	+	..	0
Sheep......................................	+	+	0	+	+	+	+	+	0
Sloth (Cholopus didactylus).............	+	+	0	0	+	+	+	+	0
Tasmanian Devil (Sarcophilus ursinus)..	+	+	0	+	..	..	+	+	0
Springbok (Gazella euchore).............	+	0	0	+	..	+	+	0	0
Tapir (Tapirella bairdi)..................	+	0	0	+	..	..	+	+	0
Zebra (Equus burchelli).................	+	0	0	+	+	0	+	+	0
Zebra (Equus zebra).....................	+	0	0	+	..	..	+	+	0

Tests by contact or layer method; results read after one hour at room temperature; + = reaction; 0 = no reaction.

enumerated. Then, in order according to the range of action, come sheep, dog, zebra, human, horse and beef precipitin serums. Here it must be noted that it was not possible to make all the tests at the same time and with antigens and antiserums of even approximately the same age. In some cases the precipitin serum was nearly two years old when some of the tests with thyroglobulins from wild animals were made, and presumably had lost much of the original strength. On the other hand, some of the thyroglobulins became more than a year old

before all the tests in table 1 were completed. Hence it would not be surprising at all if more recent antiscrums and fresher thyroglobulins would give more positive results than shown in table 1.

While it is true that in the first tests (Hektoen and Schulhof, 1923) beef and swine thyroglobulins appeared to be species-specific in their precipitin reactions, the subsequent observations show clearly that such is not always the case. And it is obvious from the results in table 1 that as prepared by us mammalian thyroglobulins are not species-specific, but more or less closely related in their precipitinogenic properties. In other words, mammalian thyroglobulin, like the proteins of the crystalline lens, seems to be organ-specific in high degree.

TABLE 2

Reactions of Simple Extracts of Thyroid Tissue with Antithyroglobulin Serums

Thyroid Extracts	Serums of Rabbits Injected with Thyroglobulin from			Control Normal Rabbit Serum
	Bear	Dog	Zebra	
Bear...........................	+	+	+	0
Beef...........................	+	+	+	0
Deer, Virginia.................	+	0	+	0
Deer, Sika.....................	+	+	+	0
Dog...........................	+	+	+	0
Goat..........................	+	+	+	0
Guinea-pig.....................	+	+	+	0
Hog...........................	0	+	0	0
Horse.........................	+	+	+	0
Rabbit........................	0	+	+	0
Raccoon.......................	0	+	+	0
Sheep.........................	+	+	+	0
Tasmanian Devil...............	+	+	+	0
Zebra.........................	+	+	+	0

+ = reaction; 0 = no reaction.

That the thyroglobulins with which we have worked exist as such in the thyroid and are not artefacts is indicated by the facts that colloid material, the lymph of thyroid lymphatics, the blood from thyroid veins, and simple extracts of thyroid tissue in salt solution all react with antithyroglobulin serum. That simple thyroid extracts are not species-specific is illustrated by table 2.

It is noteworthy that extract of rabbit thyroid and rabbit thyroglobulin may react with the serum of rabbits injected with thyroglobulin. Elsewhere has been reported the development of thyroglobulin precipitins in rabbits from the injection of rabbit thyroglobulin (Hektoen and Schulhof, 1925)—a result that finds its analogy in the production by rabbits and guinea-pigs of antibodies for the homologous lens.

It has been our experience that in general antithyroglobulin serum varies more or less in its titer with respect to different thyroglobulins. The rule seems to be that the titer of a given serum is highest for the thyroglobulin that served as antigen. In several instances, however, different serums seemed to have approximately the same titers for the same thyroglobulin. Thus precipitin serums for bear, deer, dog and zebra thyroglobulins gave the following titers, respectively, with dog thyroglobulin: 128,000, 64,000, 128,000, 64,000. These figures give the highest dilutions of dog thyroglobulin with which definite reactions developed after contact with the antiserum for one hour at room temperature.

TABLE 3

ABSORPTION EXPERIMENTS WITH SERUM AGAINST THYROGLOBULIN OF ZEBRA
(Serum, 1 Part. Thyroglobulin Solution, 2 Parts)

Thyroglobulins	Antizebra Serum + Solution of Thyroglobulin from								Control Unabsorbed Serum
	Anteater	Baboon	Bear*	Chicken	Deer† Sika	Horse	Tapir	Zebra	
Anteater...................	0	0	0	+	0	0	0	0	+
Baboon.....................	0	0	0	+	0	0	0	0	+
Bear, grizzly..............	0	0	0	+	0	0	0	0	+
Bear, polar...............	0	+	0	+	0	0	0	0	+
Bear, brown...............	0	0	0	+	0	0	0	0	+
Chicken...................	0	0	0	0	0	0	0	0	0
Deer, Virginia............	+	0	0	+	0	+	0	0	+
Deer, Sika................	0	0	0	+	0	+	0	0	+
Horse.....................	0	0	0	+	0	0	0	0	+
Tapir.....................	0	+	0	+	0	+	0	0	+
Zebra.....................	0	0	0	+	0	0	0	0	+

+ = reaction; 0 = no reaction.
* The same results also with thyroglobulins of polar and brown bear.
† The same results also with thyroglobulin of Virginia deer.

A number of absorption experiments have been made. Working with a strong serum against human thyroglobulin it was found that the precipitins it contained for beef and swine thyroglobulins were removed completely by specific absorption in each case without notable loss in precipitin for human thyroglobulin. Absorption with human thyroglobulin, on the other hand, resulted in the complete removal of all precipitins. This result seemed to point to the existence of a main, species-specific antigen and of lesser antigens of the nature of thyroglobulins in other species. In a larger series of absorption tests with an antizebra serum (table 3), not only the zebra thyroglobulin but no less than seven of the eleven heterologous thyroglobulins that were used completely deprived the serum of all precipitins; of the four other heterologous thyroglobulins, that from chicken had no effect at all, while the remaining three removed the precipitins for all the thyroglobulins included in

the experiment, and including the homologous, with from one to three exceptions. The results in this case point to a close antigenic relationship on part of the mammalian thyroglobulins concerned. It is obvious that further studies are necessary before more definite statements can be made.

In our absorption experiments two parts of thyroglobulin solution (of varying strengths) were mixed with one part of antiserum and placed in the ice-box over night, the clear supernatant fluid being used for tests the following day.

It is especially necessary as well as difficult to exclude the possibility that lipoids combined with the thyroglobulin may interact. It is probable that lipoids to some extent are removed with the euglobulin fraction. The failure of our serums against mammalian thyroglobulins to react with chicken thyroglobulin would seem to speak in general against the lipoid nature of the antigenic group common to the mammalian thyroglobulins. Precipitin serums for dog, bear and zebra thyroglobulin did not react with extracts of dog lymph node, lung, kidney, liver and salivary glands, prepared by the same method (Oswald's) that was in preparing thyroglobulin. None of the antiserums tested reacted with crude extracts from dog kidney, with the acetone insoluble and cholesterolized antigen used in the Wassermann reaction or with a 1% suspension of lecithin. Methyl alcohol extracts of the thyroglobulins have contained, so far, too little lipoid to be of any size. These are reasons why we are inclined to regard thyroglobulin as organ specific antigen in our experiments.

SUMMARY

Mammalian thyroglobulins are more or less closely related in precipitinogenic properties. Whether specific and common antigens occur in mammalian thyroglobulins, prepared according to Oswald's method, remains an open question. Apparently there is no antigenic relationship between the mammalian thyroglobulins that have been studied and the thyroglobulin of the domestic fowl.

THE PREPARATION AND PRECIPITIN REACTIONS OF EGG ALBUMIN AND BLOOD PROTEINS OF THE DOMESTIC FOWL

LUDVIG HEKTOEN AND ARTHUR G. COLE

From the John McCormick Institute for Infectious Diseases and the Laboratory of Physiological Chemistry, University of Illinois College of Medicine, Chicago

The first application of the precipitin reaction to the study of the relationship between the proteins of the egg white and blood of the fowl was made by Uhlenhuth.[1] In his first paper, he reported that intraperitoneal injections of hen's blood gave an antiserum which precipitated highly diluted fowl blood but not the egg white of the fowl. In a later paper,[2] however, he concluded that the egg whites of the hen, goose, duck and pigeon have the same albumin and that this protein is also present, to some extent, in the blood of these birds. Levene[3] reported that animals injected with egg white of the fowl gave an antiserum which formed precipitates with chicken serum, and the work of Gengou[4] indicated that the serum of rabbits injected with either egg white or defibrinated fowl blood is capable of precipitating both substances. Similar results are reported by Nuttall[5] and Galli-Valerio.[6] Nuttall's results are especially interesting because he described a great many experiments of this nature and emphasized the fact that antiserum for one antigen reacts with the other antigen only when exceedingly powerful. Even then, the reactions are always much more pronounced with the corresponding antigen.

All of this early work was with the whole egg white and either serum or defibrinated blood and, as far as we know, no attempts have been made to repeat the experiments with purified proteins. In this paper we report results of the precipitin reactions of the various proteins of fowl plasma with the results of some experiments with various fractions of egg albumin. Although some of the latter experiments are being repeated with samples of egg albumin prepared by methods other than the one reported here and the work extended to include some of the

[1] Deutsch. med. Wchnschr., 1900, 16, p. 734.
[2] Ibid., 1901, 17, p. 260.
[3] Med. News, 1901, 79, p. 981.
[4] Ann. de l'Inst. Pasteur, 1902, 16, p. 734.
[5] Blood Immunity and Blood Relationship, 1904.
[6] Ztschr. f. Immunitätsf. u. exper. Therap., 1911, 9, p. 313.

other proteins of egg white,[6a] we include the results obtained so far because of some interesting questions that arise concerning the purification of egg albumin.

PREPARATION OF BLOOD PROTEINS

Method 1.—Fibrinogen: The fibrinogen[7] was precipitated by mixing the clear plasma with one fourth volume of saturated ammonium sulphate solution; the precipitate was removed by centrifugalization, washed in 20% saturated ammonium sulphate solution and dissolved in 2% sodium chloride solution. It was then reprecipitated by ammonium sulphate and dissolved again, the precipitation and solution being repeated four times. Finally it was dissolved in 2% sodium chloride solution and dialyzed against distilled water in a collodion bag under toluol until free from both sulphate and chloride ions. The fibrinogen,

Chart 1.—Preparation of the Proteins of Chicken Plasma and Serum

Clear plasma, raised to 20% saturation of ammonium sulphate by the addition of one fourth volume of saturated ammonium sulphate solution

Precipitate of Fibrinogen	Filtrate, raised to 33% $(NH_4)_2SO_4$
	Precipitate of Euglobulin	Filtrate raised to 46% $(NH_4)_2SO_4$
	Precipitate of Pseudoglobulin	Filtrate raised to 64% $(NH_4)_2SO_4$
	Precipitate of albumin-rich fraction	Filtrate, saturated with solid $(NH_4)_2SO_4$
	Precipitate of Albumin

precipitated by this dialysis, was removed by centrifugalization, washed with distilled water and dried in thin layers on glass plates exposed to the air.

For the preparation of the serum proteins, the method used was essentially that described by Hektoen and Welker.[8]

Euglobulin: Euglobulin was precipitated in the clear filtrate from the fibrinogen by adding saturated ammonium sulphate solution to saturate the mixture 33%. It was freed as far as possible from the pseudoglobulin, always associated with it, by dissolving the protein in 10% sodium chloride solution, and reprecipitating it by dialyzing the solution against distilled water until free from chloride ions. This process of solution and dialysis was repeated until the supernatant liquid, on completion of dialysis, no longer gave a test for protein. The precipitate was then moistened with 10% sodium chloride solution and evaporated to dryness at 40 C.

[6a] See Wells, H. G.: J. Infect. Dis., 1911, 9, p. 147. The work of Wells and others on the proteins of eggwhite will be discussed in a later article.

[7] Howell, W. H.: Harvey Lectures, 1916-1917, p. 272. McLean, J.: Bull. Johns Hopkins Hosp., 1920, 31, p. 453.

[8] J. Infect. Dis., 1924, 35, p. 295.

Pseudoglobulin: This protein was precipitated in the clear filtrate from the euglobulin by adding sufficient saturated ammonium sulphate solution to saturate the mixture 46% with ammonium sulphate (chart 1). It was purified by dissolving the protein in distilled water, dialyzing against distilled water until free from sulphate ions and then reprecipitating by raising the solution on successive days to 33 and then to 46% saturation with ammonium sulphate. This process of solution, dialysis and reprecipitation was repeated until the solution no longer gave a precipitate when dialyzed free from sulphate ions. It was then evaporated to dryness at 40 C.

The filtrates from each reprecipitation of the pseudoglobulin were raised to 64 and then to 100% saturation with ammonium sulphate. The precipitates formed at both these points were also dissolved in distilled water, dialyzed against distilled water until free from sulphate ions and then evaporated to dryness.

The precipitate formed when the filtrate from the pseudoglobulin was raised to 64% saturation with ammonium sulphate was filtered, dissolved in distilled water and dialyzed against distilled water until free from sulphate ions. The solution was filtered from the precipitate formed on dialysis and the clear filtrate raised, on successive days, to 33, 46, 64 and 100% saturation with ammonium sulphate. The precipitates formed at each stage of saturation were filtered off, and those formed at 46, 64 and 100% saturation of ammonium sulphate were dissolved in distilled water and dialyzed against distilled water until free from sulphate ions. The 33-46 and 64-100 fractions were evaporated to dryness while the 46-64 fraction was reprecipitated in the fashion described, the process being repeated in its entirety three times. After the last precipitation, the solution no longer gave a precipitate when dialyzed free from sulphate ions and it was evaporated to dryness.

Albumin: This protein was precipitated in the filtrate from the precipitate formed at 64% saturation of ammonium sulphate by saturating the solution with the solid salt. It was purified by exactly the same process as that described for the 46-64 fraction except that in this case, the 64-100 fraction was reprecipitated three times while the 33-46 and 46-64 fractions were dried after the first dialysis. During the third reprecipitation of this 64-100 fraction, practically no precipitates formed when the solution was brought to 46 and to 64% saturation of ammonium sulphate, and therefore it, too, was evaporated to dryness after being dialyzed free from sulphate ions.

Method 2.—The procedure followed in this case was essentially the same as that already described, with the following exceptions. The filtrate from the fibrinogen was treated with sufficient saturated ammonium sulphate solution to saturate the mixture 50%, and after standing over night, the precipitate was filtered off and drained as much as possible. It was then covered with half saturated ammonium sulphate solution and again drained as completely as possible. The precipitate was then dissolved in the required quantity of water, and the volume noted. Following Sörensen,[9] the measured increase in volume was considered to be due to 50% saturated ammonium sulphate solution, upon which assumption the ammonium sulphate concentration of the solution may be approximated. The solution was then mixed with saturated ammonium sulphate solution to bring the mixture to 25% saturation. After standing over night, the precipitate formed was filtered off and the clear filtrate was raised to 50% saturation of ammonium sulphate. The process was repeated with the new precipitate, the precipitation between 25 and 50% saturation of ammonium sulphate being carried out four times. The protein was then dissolved in dis-

[9] Compt. rend. de Trav. du Lab., Carlsburg, 1925, 15, article 11.

tilled water and dialyzed against distilled water until free from sulphate ions, when a precipitate of euglobulin was formed. This precipitate and the supernatant liquid containing much of the pseudoglobulin were each purified in the manner previously described, the purification being repeated until the euglobulin gave no water soluble protein when dialyzed free from chloride ions and the pseudoglobulin gave no precipitate when its solution was dialyzed free from sulphate ions.

The fraction of blood protein precipitating between 50 and 64% saturation of ammonium sulphate was treated in a similar manner. It was dissolved in a measured quantity of water, the increase in volume noted being now attributed

Chart 2.—Fractionation of Egg Albumin

Egg white thoroughly mixed with an equal volume of saturated
ammonium sulphate solution

Precipitate of "Globulin"	Filtrate, treated with 10% Acetic Acid
Precipitate, P	Filtrate, F_1, treated with $(NH_4)_2SO_4$ solution
Precipitate, F_1P	Filtrate, F_2, treated with $(NH_4)_2SO_4$ solution
Precipitate, F_2P	Filtrate, F_3, treated with $(NH_4)_2SO_4$ solution
Precipitate, F_3P	Filtrate, F_4, treated with $(NH_4)_2SO_4$ solution
Precipitate, F_4P	Filtrate, F_5, saturated with solid $(NH_4)_2SO_4$
Precipitate, F_5P	

to 64% saturated ammonium sulphate solution. It was then reprecipitated, the process being repeated four times upon the fraction precipitating between 50 and 64% saturation of ammonium sulphate. The protein was finally dissolved in distilled water, dialyzed against distilled water until free from sulphate ions and then evaporated to dryness.

PREPARATION OF PROTEINS OF EGG WHITE

The crystalline egg albumin was prepared by the method of Hopkins[10] from eggs about 24 hours old. The whites were first carefully separated from the yolks and then thoroughly mixed with an equal volume of saturated ammonium sulphate solution. After standing over night, the mixture was filtered from the bulky precipitate and to the filtrate 10% acetic acid was added drop by drop

[10] J. Physiol., 1899, 25, p. 306.

with constant stirring until a slight permanent precipitate was formed. To this mixture, 1 cc. excess acid was added for every 100 cc. of original filtrate, causing the formation of a voluminous precipitate, after which the bottle was stoppered and set aside at room temperature. In about a week, this precipitate (P in chart 2) consisted wholly of rosettes of needles. It was filtered off and the filtrate (F_1) was fractionated further in a manner similar to that described by Osborne and Campbell.[11] It was first treated with saturated ammonium sulphate solution until a fairly bulky precipitate was formed, which after standing for a week, also consisted of rosettes of needles. This precipitate (F_1P) was filtered off and the filtrate (F_2) was treated with saturated ammonium sulphate solution until a third precipitate (F_2P) was formed. This process was repeated until five such fractions (P, F_1P, F_2P, F_3P, F_4P) were precipitated (chart 2). The filtrate (F_5) from this last precipitate contained so little protein that it was saturated with solid ammonium sulphate, giving precipitate F_5P. Each of the six fractions was purified by dissolving it in distilled water and adding saturated ammonium sulphate solution until a bulky precipitate was formed. These precipitates were set aside for about a week, with occasional stirring, when they were filtered off, again dissolved in distilled water and reprecipitated by the addition of ammonium sulphate solution. These processes of solution and precipitation were repeated four times with each fraction after which the precipitates were dissolved in distilled water and their solutions dialyzed, in collodion bags under toluol, against distilled water. When dialyzed free from sulphate ion, each of these solutions was raised to 50% saturation of ammonium sulphate by thorough mixing with an equal volume of saturated ammonium sulphate solution. Any precipitate formed after standing over night was filtered off and the clear filtrates were raised on successive days to 64% and then to 100% saturation of ammonium sulphate. All precipitates formed at both these points were dissolved in distilled water, filtered and the clear solutions dialyzed against distilled water until free from sulphate ion. The solutions were then evaporated to dryness at 40 C.

Of the six main fractions of egg albumin, P and F_1P consisted of rosettes of needles while the others showed only spherules under the microscope. These fractions were resolved into two components as described, one precipitating between 50 and 64% and the other between 64 and 100% saturation of ammonium sulphate, for purposes of comparison with the corresponding fractions of blood protein. The amount of protein precipitating in each fraction was variable, due probably to the fact that no attempts were made in these experiments to regulate the hydrogen ion concentration of the solutions. This factor, as shown by Sörensen,[12] exerts a marked influence on the amount of protein precipitating at any given ammonium sulphate concentration.

IMMUNIZATION OF RABBITS AND PRECIPITIN TESTS

Solutions of albumin and pseudoglobulin were prepared for use by first moistening a weighed sample of protein with physiologic salt solution and permitting it to stand until a gelatinous mass was formed. The solutions were then made up to the desired volume with salt solution, the protein usually going into solution readily. The solutions used were either 0.5 or 1% in strength.

[11] J. Am. Chem. Soc., 1900, 22, p. 422.
[12] Compt. rend. des. Trav. du Lab., Carlsberg, 1917, 12, p. 240.

Solutions of fibrinogen and euglobulin were prepared by the same method, the solvents being 2% sodium chloride and 10% sodium chloride respectively. The solutions were then filtered through cotton and the protein contents of the solutions checked by nitrogen determinations by the Kjeldahl micromethod.

Rabbits were injected intravenously with increasing doses of the solutions of blood albumin, euglobulin and various fractions of egg albumin prepared as just described. No attempt has been made in connection with the work now reported to immunize with blood pseudoglobulin. The usual amounts injected were 1, 2, 4, 6 and 8 cc. at intervals of three days. In some cases injections of larger quantities on three successive days were given with good results. The precipitin content of the serum was tested on the fourth day after the last injection, and when indicated the animal was bled at once. The precipitin tests were made by the contact method with undiluted serum and progressive dilutions of the protein solutions in 0.9% salt solution and the results read after one hour at room temperature. Comparative tests showed that fibrinogen solutions gave the same results whether the dilutions were with salt solution or 1% citrate solution. The approximate titers of the precipitin serums were determined by finding the highest dilutions of the antigens with which definite precipitates would form under the circumstances stated. In our tables the approximate titers are indicated by signs (0 to $+ + + +$) which are explained in the table footnotes.

RESULTS AND DISCUSSION

The results are illustrated in the tables. Table 1, which illustrates the results with antifibrinogen serums and the blood proteins prepared by method 1, shows that the antifibrinogen serum reacts also with euglobulin and pseudoglobulin, but not with albumin. The reaction with euglobulin and pseudoglobulin may be explained as due to admixture of these substances with the fibrinogen used as antigen, or due to the presence of fibrinogen in the euglobulin or pseudoglobulin solutions or both, or as due to both these possibilities. A new set of proteins was prepared according to method 2 in the hope of settling this question more conclusively. It is seen that in this case (table 2) those plasma proteins that do not react with serum against euglobulin also do not react with serum against fibrinogen. We are inclined, therefore, to the belief that the reactions of the plasma proteins with serum against fibrinogen are due to the presence of some fibrinogen in the euglobulin, or vice versa. We are led to this conclusion by the fact that pseudo-

globulin, which, according to our results and to those recently obtained by Sörensen [9] in his elaborate researches on the chemistry of the globulins, it is practically impossible to obtain free from euglobulin, gave a high reaction whereas albumin, easily freed from euglobulin, gave no reaction with serum against fibrinogen. This conclusion is also borne out by some results, not included in these tables, of tests made with various fractions of protein obtained during the purification of the main fractions. In each of these cases, the reactions with antifibrinogen serum

TABLE 1

PRECIPITIN REACTIONS WITH ANTIFIBRINOGEN SERUM OF BLOOD PROTEINS OF CHICKEN PREPARED BY METHOD 1

Proteins	Antifibrinogen Serum
Fibrinogen	+++++
Euglobulin	++++
Pseudoglobulin	+++
46–64 fraction	+++
Albumin	0
Chicken serum	++

TABLE 2

PRECIPITIN REACTIONS OF PROTEINS FROM CHICKEN BLOOD PREPARED BY METHOD 2

Antigens	Serum of Rabbits Injected with		
	Albumin	Fibrinogen	Euglobulin
Fibrinogen	0	+++++	++++
Euglobulin	0	++++	++++
Pseudoglobulin	0	+++	++++
50–64 fraction	++++	0	0
Albumin (64–100)	++++	0	0
50–64 fraction from 64–100	++++	0	0
Chicken serum	+++	++	++

0 = no reaction, + = titer of 10 to 100, ++ = titer of 100 to 1,000, +++ = titer of 1,000 to 10,000, ++++ = titer of 10,000 to 100,000, +++++ = titer of 100,000 +.

decreased or vanished, depending upon the completeness with which the proteins were freed from euglobulin.

While the method of preparing the fibrinogen would seem to exclude euglobulin in the final solution of fibrinogen, any fibrinogen not precipitated when the plasma is first raised to 20% saturation of ammonium sulphate would appear in the other protein fractions. In that case, it would be found associated with euglobulin and it would be impossible to separate them by the method used because of their similar properties of solubility in salt solution and precipitation by dialysis. Our results, therefore, are probably best explained upon the assumption that the euglobulin solutions used in our experiments contain some fibrinogen. This is indicated, also by the fact that when fibrinogen solution is

treated with the euglobulin antiserum, all of the fibrinogen may be taken out. It is of interest, in this connection, to note that one of our euglobulin solutions, which at first reacted with antifibrinogen serum, lost this property after standing in the ice box for 3 or 4 weeks. Whether this was due to changes in the euglobulin solution or in the antiserum remains undetermined. It is also of interest that fibrinogens from duck,

TABLE 3

Precipitin Reactions of Albumin Fractions of Egg White with Serums Against Albumin, Euglobulin and Fibrinogen of Chicken Blood

Antigens	Serum of Rabbits Injected with		
	Blood Albumin	Fibrinogen	Blood Euglobulin
Eggwhite Albumins:			
P, (46–64).........................	++++	0	0
F₁P.............................	+++	0	0
F₂P, (46–64).....................	++	0	0
F₃P.............................	++++	0	0
F₄P.............................	++++	0	0
F₅P, (64–100)...................	++	0	0
Blood Proteins:			
Albumin.........................	+++++	0	0
Fibrinogen......................	0	+++++	++++
Euglobulin......................	0	++++	++++

TABLE 4

Precipitin Reactions of Albumin Fractions of Egg White

Antigens	Serum of Rabbits Injected with						
	P	F₁P	F₃P	F₅P	Blood Albumin	Blood Euglobulin	Blood Fibrinogen
Egg Albumins:							
P...............	+++++	++++	++++	++++	++++	0	0
F₁P.............	++++	++++	++++	++++	++	0	0
F₃P.............	++++	++++	++++	++++	++	0	0
F₅P.............	++++	+	++++	++++	0	0	0
Blood Proteins:							
Albumin 1........	+	+	+	+	+++	0	0
Albumin 2........	++	++	++	++	+++	0	0
Euglobulin.......	0	0	0	0	0	++++	++++
Fibrinogen.......	0	0	0	0	0	++++	+++++
Chicken serum....	+	+	+	+	+++	++	++

0 = no reaction, + = titer of 10 to 100, ++ = titer of 100 to 1,000, +++ = titer of 1,000 to 10,000, ++++ = titer of 10,000 to 100,000, +++++ = titer of 100,000 +.

goose, guinea-hen and turkey appear to react with serum against chicken fibrinogen but not with serum against chicken euglobulin.

Table 3 gives the results of the tests of the albumin fractions of egg white with serums against the albumin, euglobulin and fibrinogen of blood plasma. The close relation of certain egg albumin fractions to blood albumin and the absence of any relation of the various egg albumin fractions to blood euglobulin and fibrinogen are evident. The

large differences in the dilutions of the various egg albumins that reacted with serums against the blood albumin suggest that we are dealing here with two or more egg albumins, thus linking our work with that of many early observers on the chemistry of egg albumin, notably Osborne and Campbell,[11] who claimed that egg albumin consists of two albumins, a crystalline ovalbumin, and a noncrystalline conalbumin. In order to test this point, rabbits were immunized against the fractions of egg albumin P, F_2P, F_3P and F_5P and the antiserums tested with each of the fractions and also the blood proteins. The results of these tests are given in table 4, which shows that each of the fractions of egg albumin react also with the serums against the others.

Although it is generally claimed that the crystalline egg albumin (ovalbumin) may be obtained free from the noncrystallizable albumin (conalbumin) the work of Sörensen [12] on the crystallization of ovalbumin indicates that only a portion of the crystalline protein in egg white may be crystallized out, the remainder remaining behind in the mother liquor as in all crystallization processes. In that case, it follows that in a fractionation like ours in which all of the coagulable albumin of the egg white was removed from solution the later noncrystalline fractions would contain some ovalbumin. This would account for the interactions of all of our fractions of egg albumin. The question whether it is possible by a more refined process of fractionation to separate the albumins of egg white into fractions which are immunologically distinct and to determine which of these fractions is responsible for the reaction with the serum against blood albumin is reserved for further work.

<div align="center">SUMMARY</div>

According to the precipitin reaction, albumin can be separated from the other principal proteins of chicken blood plasma by ammonium sulphate precipitation and dialysis, but it seems that this method is incapable of bringing about a complete separation of euglobulin and pseudoglobulin. It is difficult also, if not impossible, to secure in this way a complete separation of fibrinogen from euglobulin.

There is a definite immunological relationship between the albumins of egg white and of chicken blood and the albumin of egg white may be resolved into several fractions which differ widely in their reactions with precipitin serum for blood albumin.

SURFACE TENSION STUDIES WITH L. ACIDOPHILUS AND L. BULGARICUS

Nicholas Kopeloff and Philip Beerman

From the Department of Bacteriology, Psychiatric Institute, Ward's Island, N. Y.

Much confusion attaches to the differentiation between L. acidophilus and L. bulgaricus. Neither the production of acidity, nor the fermentation of maltose, sucrose and levulose have proven adequate criteria for establishing the identity of these lactobacilli.[1] Recently Albus and Holm [2] suggested the surface tension method for distinguishing culturally between the two closely related organisms under discussion. In a brief note we [3] corroborated the finding that in a medium whose surface tension is depressed by sodium ricinoleate, L. bulgaricus was inhibited at a surface tension below which L. acidophilus developed readily. The subject appeared to be worthy of further investigation, the results of which are here reported.

Methods.—The procedure used by Albus and Holm,[2] which was kindly placed at our disposal by them before publication, has been modified in an attempt to establish a standardized method which would permit of easily repeated tests falling within the critical range.

The medium for growth is made up of 1% yeast, 1% peptone, 1% lactose, 1% beef extract and 0.3 cc. of a 5% alcoholic solution of brom cresol purple per liter, as an indicator. The yeast is digested by autoclaving 40 gm. of yeast in one liter of distilled water for 2 hours at 20 lbs. pressure. The proper amounts of other ingredients are added to the digest which is made up to 4 liters and autoclaved for 15 minutes at 10 lbs. pressure. It is allowed to stand in the ice box for 24 hours after which the supernatant liquid is decanted and resterilized for 15 minutes at 15 lbs. pressure. The final P_H should be about 7.0.

All cultures to be used are acclimatized to this medium by 3 successive daily transfers. When satisfactory growth is obtained they are ready for inoculation into the same medium to which depressants are added in the following manner, taking the desirable concentration of 4.2% sodium ricinoleate as an example.

Especially purified sodium ricinoleate very generously placed at our disposal in liberal quantities by Dr. W. P. Larson of the University of Minnesota, is made up by weighing out 4.4 gm. sodium ricinoleate, adding it to 100 cc. distilled water and thoroughly dissolving. The solution is filtered through paper. With a pipet amounts of 0.5 cc., 1.0 cc., 2 cc., 5 cc., etc., are each diluted to 200 cc. with the yeast medium as diluent. When different percentages of sodium ricinoleate are used, i. e., 0.275, 0.6, 2.1, 6.1, etc., the same method of dilution of 0.5, 1.2 cc. etc. to 200 cc. of yeast medium is followed. Similarly with sodium

[1] Kopeloff, N.: Lactobacillus Acidophilus, 1926.
[2] J. Bact., 1926, 12, p. 13.
[3] Proc. Soc. Exper. Biol. & Med., 1926, 23, p. 544.

oleate except that when high percentages such as 23.4 are used the graded amounts, 1.2 gm., 1.8 gm., 2.4 gm. etc., are weighed directly into 200 cc. of yeast medium. The depressed mediums, in 6 cc. amounts are then sterilized in Pyrex test tubes for 15 minutes at 15 lbs. pressure. After cooling, the tubes are inoculated in duplicate with 0.20 cc. of an acclimatized culture. Triplicate uninoculated tubes are kept in the ice box while duplicate uninoculated and inoculated tubes are incubated as controls with the inoculated tubes at 37 C. for 7 days. Tubes in which growth occurs change in color from purple to yellow.

To measure the surface tension of the above solutions, a Traube stalagmometer has been employed. Obviously, great care must be taken to keep this instrument clean. In order to carry out the determinations at a constant temperature the stalagmometer, distilled water, beakers, etc. are kept in a small inoculation room regulated at 25 C. for at least 30 minutes prior to working and the solutions are brought to this temperature when tested.

Procedure.—To the upper end of the stalagmometer is attached a rubber tube approximately 220 mm. by 3.5 mm. with a glass mouthpiece. A screw pinchcock is used to regulate the flow of drops. A small beaker containing distilled water is placed under the lower end of the stalagmometer and the water is sucked up to within one inch of the upper end. After washing out 2 or 3 times, the stalagmometer is almost filled with water and the drop surface dried off with lens paper. The pinchcock is so regulated that the drops flow at the rate of about one every 4 to 6 seconds. It is very important to time this rate as more than 18 to 20 drops per minute should not be allowed.

One of the triplicate, uninoculated tubes of the medium (kept in icebox), depressed with a given amount of depressant is used to rinse out the stalagmometer several times. Previously the solution should be carefully decanted into a small clean beaker in every instance so as to leave behind the sediment or precipitate. This procedure is more desirable than filtration for it precludes the possible absorption of depressant. Then readings of the number of drops delivered between 2 marked points on the stalagmometer are made on the solutions to be tested (uninoculated or inoculated). Thus, water which should always be run as a control gives a reading of 22.5 drops at 25 C. with our stalagmometer while 0.5 cc. of 4.2% sodium ricinoleate in 200 cc. of medium gives readings averaging 37.5 drops. It should be stated that in general the duplicates on uninoculated medium agree within 0.5 drop while duplicates on inoculated medium agree within 2 to 3 drops. When dealing with inoculated tubes the supernatant liquid should be pipetted into a small clean beaker taking care not to disturb the floating scum or the deposit.

Having determined the number of drops delivered by the various depressed solutions and the specific gravity of the yeast medium by the usual pycnometer method, the following formula is used for calculating in dynes:

$$\frac{72.1}{X} = \frac{\text{number of drops of solution} \times 1.0}{\text{specific gravity of solution} \times \text{number of drops } H_2O}$$

72.1 = number of dynes per cm. (surface tension of water).
Number of drops of solution, or H_2O = number of drops of the fluid as delivered by stalagmometer.
1.0 = approximate specific gravity of water.
       Specific gravity of solution is determined by pycnometer.
X = number of dynes per cm. (surface tension of solution).

Cultures.—The cultures used in this investigation have come from several sources. A1 represents an X strain of L. acidophilus, originally obtained from Dr. W. L. Kulp of Yale University, which we have repeatedly passed through the human intestine with marked therapeutic results.[1] We are also indebted to the same investigator for L. bulgaricus strains B1, B2 and B3. L. acidophilus cultures A2 and A3 as well as L. bulgaricus B4 and B5 were employed by Albus and Holm[2] in their surface tension experiments and were kindly offered to us. L. acidophilus Y strains, A4 and A5 are being utilized commercially. L. acidophilus Y strains, A6 and A7 are also employed commercially but have not been experimentally investigated by us. The same is true of L. acidophilus strain A8.

Laboratory methods for the differentiation of lactobacilli are of importance but in the last analysis our present interest in these organisms centers about their therapeutic application. L. acidophilus survives in the human intestinal tract and can induce a transformation of the intestinal flora under proper conditions. L. bulgaricus cannot.[1] Therefore no matter how the organism be labelled we must rely ultimately upon human passage to establish its identity. This has been authenticated in the case of our L. acidophilus strains A1, A4 and A5. The original source of the other L. acidophilus strains employed is such as to leave no room for question but second-hand cultures should not be greatly relied upon in fundamental work.

The L. bulgaricus cultures, B1 and B4 have been employed without obtaining implantation in feeding experiments, by Kulp and Rettger[4] and ourselves,[1] thus assuring the identity of these strains.

Results.—Using the cultures and procedure described above the results obtained with sodium ricinoleate as depressant are recorded in table 1.

Under each percentage (0.275, 0.6, 4.2, 4.4 and 6.1), in the first column, are given the lowest number of dynes at which growth occurred. In the second column, are given the highest number of dynes at which growth did not occur. There is little point in tabulating here higher values where growth has occurred, or lower values at which it has not occurred, except where it narrows the difference so that a critical point can be established. In general each set of "lowest" and "highest" figures here presented represents the critical points arrived at in a series of approximately 6 to 10 different concentrations. The space required for our complete data seems scarcely justifiable, consequently they have been

[4] J. Am. M. A. 1926, 87, p. 833.

omitted. A simple illustration will suffice: culture A1 was tested in 4.4% sodium ricinoleate using 0.1, 0.2, 0,3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 cc. per 200 cc. of medium. Growth occurred at 0.1, 0.2, 0.3, 0.4, 0.5 cc. but did not occur at 0.6, 0.7, 0.8, 0.9 and 1.0 cc. Therefore the surface tensions of only 0.5 and 0.6 cc. were determined. These were found to be 36.1 and 35.0 dynes respectively. Subsequent repetition narrowed this range to between 35.3 and 35.0 dynes. Most of the sets of "lowest +" and "highest 0" figures do not represent such a narrow range. For example, in table 1 the first set of figures for A1

TABLE 1

Growth of L. Acidophilus and L. Bulgaricus at Varying Surface Tensions (in Dynes) with Sodium Ricinoleate as Depressant

	0.275%		0.6%		4.2%		4.4%		6.1%			
	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0
L. acidophilus												
A 1.......	40.2	....	39.3*	....	39.4 39.2	34.1 34.7	39.2 37.8 36.1 35.3	34.5 35.0	39.4	34.7	35.3	35.0
A 2.......	....	....	39.3*	....	39.2	35.0	....	....	39.4	36.5	39.2	36.5
A 3.......	....	....	39.3*	....	....	....	....	....	42.7	39.4	39.3	39.4
A 4.......	....	....	....	....	39.4	34.5	39.2*	34.5*	....	....	39.2	34.5
A 5.......	....	....	....	....	36.1	35.0	....	....	....	....	36.1	35.0
A 6.......	....	....	....	....	39.2	38.1	....	....	....	....	39.2	38.1
A 7.......	....	....	....	....	39.2	38.1	....	....	....	....	39.2	38.1
A 8.......	....	....	....	....	42.6	39.4	....	....	....	....	42.6	39.4
L. bulgaricus												
B 1.......	44.7 44.7	42.9 43.5	....	....	43.5	40.7	....	....	....	....	43.5	42.9
B 2.......	43.5	....	43.5	42.6	51.2* 44.7 44.5	42.3 42.6 44.4	....	....	....	....	43.5	42.6
B 3.......	....	....	....	....	43.5	39.2	....	....	....	....	43.5	39.2
B 4.......	....	....	....	....	....	43.5	....	....	....	....	....	43.5

* Repeated.

Figures indicate reading of highest tension with no growth (0), and lowest tension allowing growth (+), with different amounts (%) of depressant. No test is indicated by .....

under 4.4% are 39.2 and 34.5 dynes respectively. This means that no intermediate values were included in this particular test, rather than that growth did not occur within such a wide range. Obviously, the trial and error method required for such work makes for duplication of tests in order to establish the critical point. In this sense the values recorded in the last two columns of table 1 are provisional and not final. It should be remembered that unless otherwise stated, all the values in dynes are based on uninoculated depressed medium. The experimental error can be considered to be about 0.5 dyne.

Examining table 1 in detail one finds that the range of "lowest +" and "highest 0" for any single percentage of sodium ricinoleate is about 3 dynes although it may be as much as 5 dynes. There is not much evidence upon which to base a comparison of different percentages of sodium ricinoleate as influencing the growth of any particular organism

TABLE 2

Growth of L. Acidophilus and L. Bulgaricus at Varying Surface Tensions (in Dynes) with Sodium Oleate as Depressant (%)

	3.4%		4.2%		8.0%		11.7%			
	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0	Low-est +	High-est 0
L. acidophilus										
							25.5			
A 1	35.7	....	35.0	....	24.0	....	25.2	24.2	24.0	24.2
A 2	....	....	35.0	....	....	....	....	....	35.0	....
A 3	....	....	36.0	....	....	....	....	....	36.0	....
A 4	....	....	35.0	....	....	....	....	....	35.0	....
A 5	....	....	35.0	....	....	....	....	....	35.0	....
A 6	35.7	....	....	....	....	....	....	....	35.7	....
A 7	35.7	....	....	....	....	....	....	....	35.7	....
A 9	....	....	40.3	38.5	....	....	....	....	40.3	38.5
L. bulgaricus										
B 1	41.2	33.5	....	....	....	....	....	28.2	35.7	33.5
	36.0									
	35.7									
B 2	48.7	43.5	43.5	42.3	....	....	....	....	48.7	43.5
B 3	....	....	43.5	42.3	....	....	....	....	43.5	42.3
B 4	....	....	43.5	42.3	....	....	....	....	43.5	42.3

TABLE 3

Critical Surface Tension for Growth of L. Acidophilus and L. Bulgaricus (in Dynes)

	Sodium Ricinoleate			Sodium Oleate		
	Lowest +	Highest 0	Critical Point	Lowest +	Highest 0	Critical Point
L. acidophilus						
A 1	35.3	35.0	35.2	24.0	23.6	23.8
A 2	39.2	36.5	37.9	35.0	....	....
A 3	39.3	39.4	39.4	36.0	....	....
A 4	39.2	34.5	36.9	35.6	....	....
A 5	36.1	35.0	35.6	25.5	....	....
A 6	39.2	38.1	38.7	35.7	....	....
A 7	39.2	38.1	38.7	35.7	....	....
A 8	42.6	39.4	41.0	36.0	....	....
A 9	....	41.2	....	40.3	38.5	39.4
Average	....	....	37.9	....	....	....
L. bulgaricus						
B 1	43.5	42.9	43.2	35.7	33.5	34.6
B 2	43.5	42.6	43.1	43.5	43.5	43.5
B 3	43.5	39.2	41.4	43.5	42.3	42.9
B 4	....	43.5	43.5	43.5	42.3	42.9
B 5	43.5	42.6	43.1	....	....	....
B 6	....	43.5	....	....	....	....
Average	....	....	43.6	....	....	41.0

but in general it does not appear to make any difference whether higher or lower concentrations are employed. Our preference is for the mean, that is neither a high or low percentage, for either extreme makes for an increase in experimental error, the high percentages because of the danger of unequal distribution, the low percentages because of increase in dilution of the medium. The difference between the growth of L acidophilus and L. bulgaricus at varying surface tensions makes it difficult to use any single percentage of sodium ricinoleate for both species. Thus from the data in table 1 the percentage of choice for L. acidophilus would be 4.2, while for L. bulgaricus it would be 0.275.

In table 2 are presented similar data where sodium oleate (c.p.) has been used as depressant. While the critical ranges have not yet been worked out for all the organisms used it will be seen that in general the nature of the results resembles that of table 1.

Of further interest is the summary presented in table 3 which permits a comparison of the critical values of the last two columns shown in tables 1 and 2. For each soap used the "Critical Point" is calculated the midpoint between the lowest and highest readings. Several interesting facts are here disclosed. It will be seen that under sodium ricinoleate the three therapeutically effective L. acidophilus cultures, A1, A4 and A5 show the lowest critical points, namely, 35.2, 36.9 and 35.6 dynes respectively. The other L. acidophilus cultures may possess therapeutic value but this has not yet been satisfactorily demonstrated. The L. bulgaricus cultures all have a higher critical point than the L. acidophilus cultures. Again it is significant that the two cultures B_1 and B_2, which in our hands have failed of intestinal implantation, had critical points of 43.2 and 43.1 respectively. In other words there is a difference of 7.3 dynes between the growth of our authentic L. acidophilus and L. bulgaricus cultures. Such a difference lies beyond any experimental errors involved and merits discussion later.

It is scarcely necessary to point out that the commercial L. acidophilus cultures (A8 and A9) which gave fairly high critical points may be regarded with considerable suspicion as being borderline strains. In any event the average of all the strains called L. acidophilus is 37.9 while that of L. bulgaricus is 43.6—a significant difference of 5.7 dynes.

Turning our attention to the values for sodium oleate in table 3 we find that the critical point for A1 is 23.8 dynes. The average critical point for the L. bulgaricus cultures with this depressant is 41.0, or a difference of over 17 dynes, which is very striking. In a later discussion we will consider the important finding that the critical point for L. acid-

ophilus is 11.4 dynes lower with sodium oleate than with sodium ricinoleate, while the corresponding values for the L. bulgaricus cultures are not so widely divergent.

In considering the growth of bacteria at varying surface tensions it has already been stated that the determinations were made on uninoculated depressed mediums. It was of interest in this connection to make a few determinations of inoculated depressed media after 7 days incubation at 37 C. The anticipation of differences of varying degrees was realized and the data are presented in table 4.

It will be seen from table 4 that with 4.2% sodium oleate the difference between inoculated and uninoculated (control) medium for two strains of L. bulgaricus was about 6.0 dynes. Similarly, in higher percentages, L. acidophilus cultures gave differences of about 8.0 dynes. With sodium ricinoleate however, the differences were very much less.

TABLE 4

Comparison of Surface Tension (in Dynes) in the Presence and Absence of Growth

| | Sodium Oleate | | | | | | | | | Sodium Ricinoleate | | |
| | 4.2% | | | 11.7% | | | 23.4% | | | 4.2% | | |
Organism	Control	Growth	Difference	Control	Growth	Difference	Control	Growth	Difference	Control	Growth	Difference
A 1	....	....	....	25.5	33.5	8.0	25.1	33.5	8.4	....	....	....
A 5	....	....	....	25.5	32.8	7.3	....	....	....	36.1	39.2	3.1
B 1	35.7	41.5	5.8	....	....	....	....	....	....	43.5	44.0	0.5
B 5	32.8	38.7	6.1	....	....	....	....	....	....	43.5	45.9	2.9

Gibbs, Batchelor and Sickels have published interesting data of a similar nature using various soaps.[5] From a practical standpoint it is much more difficult to deal with inoculated than with uninoculated medium in making stalagmometer readings, for the bacterial growth not only dirties the apparatus but also affects the flow of drops. Furthermore, it is to be expected that the production of lactic and other acids would influence the depressant.

Since surface tension appears to be an adequate criterion for differentiating L. acidophilus from L. bulgaricus it is of interest to compare it with the most satisfactory previous method, namely, the fermentation of sugars. Therefore from active milk cultures transfers were made to sugar-free casein digest broth, P_H 7.0, containing brom cresol purple as an indicator. Sterilization without heat after adding the sugars was effected through Mandler diatomaceous filters. Dextrose was used chiefly as a control to determine growth since it is fermented by the

[5] J. Bact., 1926, 11, p. 393.

lactobacilli. The sucrose used by us was first tested by B. coli which does not ferment this sugar. All controls were negative after 48 hours incubation while the results obtained with inoculated tubes are presented in table 5, together with the critical surface tension expressed in dynes, using sodium ricinoleate as depressant.

It will be seen from table 5 that the only culture to ferment all the sugars was A1. It also grew at the lowest surface tension. The other two authentic L. acidophilus strains, A4 and A5 fermented maltose, levulose and dextrose but not sucrose. They were able to grow at very low surface tensions, indicating that this test is superior to sugar fermentation in establishing the identity of the culture. L. acidophilus A6 and A7 grew at a lower surface tension than any L. bulgaricus strains

TABLE 5

Sugar Fermentation and Surface Tension Growth

	Critical Point in Dynes (Ricinoleate)	Dextrose	Sucrose	Maltose	Levulose
A 1................	35.2	+	+	+	+
A 2................	37.9	+	+	+	+
A 4................	36.9	+	0	+	+
A 5................	35.6	+	0	+	+
A 6................	38.7	+	0	+	0
A 7................	38.7	+	0(?)	+	+(?)
A 8................	41.0	+	+	0	0
A 9................	39.4*	+	0	0	0
B 3................	41.4	+	0	0	0
B 4................	43.5	+	0	0	0
B 5................	43.1	+	0	0	0
B 6................	43.5	+	+	+	+

* Oleate.
Fermentation (+), and no fermentation (0), respectively.

yet the former failed to ferment either sucrose or levulose and the latter only feebly attacked levulose. Again this points to the reliability of the surface tension test as compared with the variable results obtained in sugar fermentation. Curiously, A8 supposedly L. acidophilus, was inhibited in growth at 41 dynes which is close to the point at which L. bulgaricus cannot grow, but definitely on the L. acidophilus side of the line of demarcation. Yet with this strain sugar fermentation is negative with maltose and levulose, again demonstrating the unreliability of the latter test. Strain A9 labelled L. acidophilus is also on the borderline shown by its failure to grow at a surface tension of 41.2 dynes with sodium ricinoleate and a critical point of 39.4 dynes with sodium oleate. This strain fails to ferment any of the sugars employed except dextrose. B6 which was labelled L. bulgaricus fermented all the sugars used but failed to grow below 43.5 dynes.

L. bulgaricus strains can only grow at higher surface tension values than L. acidophilus. All the strains of L. bulgaricus we employed failed to ferment any of the sugars used except dextrose.

A survey, therefore, of the data in table 5 leads to the conclusion that the surface tension test is much more reliable than sugar fermentation because of the variability of the latter. The surface tension test agrees more closely with clinical trials at implantation. Consequently it is the method of choice from a laboratory standpoint and as a possible substitute for exhaustive clinical investigations.

DISCUSSION

From the foregoing data it is evident that surface tension offers a satisfactory criterion of differentiation between L. acidophilus and L. bulgaricus of known authenticity. In all we have dealt with 12 cultures so that any generalizations must be necessarily limited. While the differentiation between the few authentic cultures is striking, there may exist borderline strains which may not be so prettily dealt with. Furthermore, the continued cultivation of lactobacilli on artificial media may exert an untoward influence sufficiently great to blur marked differentiation. But consistent results with each organism have been obtained and in general these agree with the findings of Albus and Holm [2] who state that "none of the strains of Lactobacillus bulgaricus showed evidence of growth when the medium employed was depressed with sodium ricinoleate to a surface tension of less than 40 dynes, and that most of the strains were inhibited well above this value while all the strains of Lactobacillus acidophilus were able to grow when the medium was depressed to a surface tension as low as 37 dynes." On the same cultures, of greater age, our results for L. bulgaricus gave an average critical point of 43.6 dynes and for L. acidophilus 38.7 dynes. The discrepancy is one of degree and not of kind.

It was stated that different critical values were obtained where different depressants were used. Albus and Holm [2] mention inconsistent results when using sodium glycocholate. Walker [6] pointed out that "soaps prepared from pure fatty acids differ markedly in their germicidal properties." In a comprehensive investigation Frobisher [7] found that "as compared with oleate, the other reducents inhibited growth irregularly, at surface tensions too high to explain their effects solely as the result of lowered tension." Similarly, Gibbs, Batchelor and

[6] J. Infect. Dis., 1924, 35, p. 557.
[7] J. Infect. Dis., 1926, 38, p. 66.

Sickels [5] have pointed out that "there is a considerable difference in various soaps, not only in their ability to depress surface tension, but in their effect on the organisms." Unfortunately, none of these interesting investigations have included sodium ricinoleate as a depressant or lactobacilli as test organisms.

Because of the fact that we have noted the growth of L. acidophilus with sodium oleate present at values considerably lower than those obtained where sodium ricinoleate was used it is possible to infer some inhibition, other than surface tension, due to the latter depressant. However, it should be remembered that this difference was scarcely manifest when L. bulgaricus cultures were employed. This might argue that sodium oleate has a greater nutritional value for L. acidophilus as compared with L. bulgaricus and thus account for growth at lower surface tensions. It seems reasonable to assume that neither sodium ricinoleate nor sodium oleate are sufficiently germicidal to these organisms to invalidate their use for surface tension tests.

A final word as to the practical value of the surface tension test for differentiating L. acidophilus from L. bulgaricus. The ultimate criterion is survival in the human intestinal tract. This necessitates the expenditure of much time and energy to say nothing of adequate cooperation of many human subjects. The surface tension test therefore, represents a shortcut which from a laboratory standpoint may take the place of the more exacting clinical trials. Sugar fermentation tests fail to perform the same function. According to the advice of Dr. W. P. Larson the surface tension of the intestinal contents approximates the critical point established for L. bulgaricus. Frobisher [7] claims that "bile reduced the surface tension to about 44 dynes." Albus and Holm [2] state: "that surface tension may be a factor in the implantation of these organisms seems a plausible assumption." In other words, the surface tension test is not only of interest as regards the physiological activities of the lactobacilli but bids fair to offer a comparatively simple method of determining therapeutic possibilities.

SUMMARY

Within the limitations of the experimental material under consideration the following points have been established. A standardized method for surface tension determinations on lactobacilli has been described and applied to 12 different strains of L. acidophilus and L. bulgaricus. With sodium ricinoleate 3 strains of L. acidophilus of proven therapeutic value

grew in a medium depressed below 36 dynes, while 2 strains of L. bul-
garicus proven by us incapable of intestinal implantation were inhibited
at 43.2 dynes. The average critical point for all cultures labelled L. acid-
ophilus was 37.9 dynes and for L. bulgaricus 43.6 dynes. With sodium
oleate L. acidophilus grew to 23.8 dynes while L. bulgaricus was inhib-
ited at 41 dynes. The surface tension of inoculated and incubated cul-
tures is higher than that of uninoculated controls kept in the ice box.

Surface tension tests on lactobacilli compare favorably with sugar
fermentation but offer a more accurate method of differentiation, agree
with clinical trials, and may possibly serve as a substitute for the latter
in determining therapeutic efficiency.

INCIDENCE OF MASTITIS AND THE INFECTING ORGANISMS IN FOUR DAIRY HERDS

John G. Hardenbergh and Carl F. Schlotthauer

From the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minn.

The data presented here concerns the occurrence of bovine mastitis in four of the larger dairy herds that have been under our observation during the last two and a half years. These herds comprise about 280 cows of milking age, and three of the herds, which include 90% of the animals under observation, are maintained under conditions of rather intensive milk production. Milking machines are used in all four herds.

Sixty-eight cases involving 65 animals have been studied. As will be shown, practically all of these cases were of the infectious type, less than 1% failing to yield infecting organisms in the milk cultures. It is probable that a considerable number of other cases of mastitis occurred in these herds but either escaped notice or were so transient that they

TABLE 1

INCIDENCE OF MASTITIS

Farm Number	Number of Cases		
	1924 (6 Months)	1925	1926
1	6	17	1
2	10	7	1
3	3	10	6
4	6	0	1
Total	25	34	9

were not reported; these probably comprise the group of noninfected or mildly infected cases which remain unrecognized except in herds in which particular pains are taken to examine the milk of each animal at each milking for the first traces of abnormal secretion as an index to disease of the udder. However, from our experience, it would seem that cases of mastitis unaccompanied by infection of some sort are not ordinarily encountered by the veterinarian. It should be noted (table 1) that the number of cases listed for 1924 was for the last six months only. It is likely that there would be reduction for 1925 over the total number of cases for the whole of 1924, if the total number were available. The marked decrease in 1926 is not to be credited to any specific

preventive measures but certainly may be due in part to greater attention to the following details of dairy hygiene. All cows with mastitis are isolated from the healthy herd if possible, and they are all milked by hand, always after the rest of the herd has been attended to. The milk and udder secretions are drawn into pails containing disinfectant solution. The attendant washes his hands before and after milking or manipulating each diseased udder. As a general prophylactic measure indiscretions of diet are avoided as much as possible, the cows are milked at regular intervals, and clean stalls and abundant litter are provided. All unnecessary trauma to the udder and teats, no matter how slight, is avoided.

Bacteriologic Examination.—Collection of Milk Samples: The precautions observed in obtaining milk samples from a diseased udder for bacteriologic diagnosis consisted in carefully cleansing the teats and under surface of the udder, followed by sponging the teat, especially the opening, with 95% alcohol. After the first few streams of milk were discarded, the samples were drawn into sterile tubes. Owing to the proximity of the farms to the laboratory, it was possible to culture the samples in most cases soon after they were drawn and no effort was made to refrigerate the samples in transit.

Cultural methods: Specimens were cultured in glucose brain broth (Rosenow [1]) and on blood agar plates. The former medium in deep tubes is particularly suited to the cultivation of streptococci but is also well adapted to the growth of a wide variety of organisms, due to the gradient of oxygen tension which exists in the tubes. Cultures were incubated for 18 to 24 hours. In the few cases not showing pure culture, single colonies of organisms were picked from the plates for identification, or the broth cultures were plated out. The various strains of streptococci isolated were classified as a routine only as to their behavior on blood agar as green-producing (alpha), hemolytic (beta), or nonhemloytic (gamma), although a few strains were studied to learn their fermentation reactions on carbohydrates. Attempts to classify streptococci strictly are, at best, likely to be only mildly successful and to obscure the main issue, that is, the type of infection present (table 2).

Results: The various organisms encountered were in pure culture in all but eight cases that have been classified as mixed infections. Streptococci were found to be the infective agent in pure culture in slightly more than half the cases, and in the cases of mixed infection streptococci were present (with staphylococci) seven times out of eight. Farm 1 showed a predominance of streptococcal infections, and of the 19 strains

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[1] J. Dent. Research, 1919, 1, p. 205.

isolated there, 12 were green-producing, four were hemolytic and three were nonhemolytic. Streptococci accounted for slightly more than half the cases on farm 2, and here the strains encountered were five green-producing, two hemolytic and three nonhemolytic. The relatively large number of staphylococcus infections on farm 2 is of interest. Farm 3 furnished all the cases of mastitis with Bacillus coli infection. All six cases occurred in one barn while, at about the same time, streptococcus infection was present in another barn on the same place. In these cases due to Bacillus coli infection the onset was sudden and the systemic reaction was marked, as in severe toxemia. Milk secretion in the unaffected quarters was greatly diminished. The diseased quarters were enlarged, firm, hot and sensitive. The secretion contained therein was at first flaky, and the flakes of curd readily precipitated on standing leaving a

TABLE 2
Organisms Isolated in 68 Cases of Mastitis

	Farm 1	Farm 2	Farm 3	Farm 4	Totals
Streptococci	19	10	7	4	40
Staphylococci	1	5	0	1	7
Bacillus coli	0	0	6	0	6
Bacillus lactis-aërogenes	0	1	0	0	1
Pasteurella boviseptica	0	0	1	0	1
Unidentified bacillus	0	0	1	0	1
Mixed infection	3	1	3	1	8
No growth	1	1	1	1	4
Total	24	18	19	7	68

clear supernatant fluid. In a few days the secretion assumed the character of thick lumpy, fetid pus, and the affected quarter of the gland became one large abscess.

Infections with Bacillus coli in the udder apparently cause rapid destruction of udder function, but are not so destructive to the udder tissue itself as staphylococcus infections, the tendency of the latter to cause digestion of tissues being marked. The case from which a bipolar organism, probably Pasteurella boviseptica, was isolated has already been reported.[2] Briefly, this case yielded on two occasions pure cultures of an organism which was indistinguishable from members of the hemorrhagic septicemia group and was virulent for rabbits, although a nursing calf showed no ill effects as a result of having drunk milk from the udder when it was first affected; and the quarter of the udder most severely affected never regained its function. The cases of mixed infection presented the following association of organisms: Staphylococcus albus and Bacillus coli, 1; Staphylococcus albus and green-producing

[2] J. Am. Vet. M. A., 1926, 69, p. 506.

streptococci, 1; Staphylococcus albus and hemolytic streptococci, 1; Staphylococcus albus and nonhemolytic streptococci, 3; Staphylococcus aureus and hemolytic streptococci, 1; Bacillus coli and green-producing streptococci, 1.

Of all the streptococci (47 strains) including those associated with other organisms, the types isolated relative to their behavior on blood agar are as follows: green-producing (alpha hemolytic type), 23; hemolytic (beta hemolytic type), 9; nonhemolytic (gamma type), 15. Of the staphylococci, including those associated with other organisms, the 14 strains were classified as Staphylococcus albus (11) and Staphylococcus aureus (3); one strain of the former was markedly hemolytic on blood agar.

DISCUSSION

In general, our bacteriologic data agree with those of other investigators who have studied mastitis. It is quite evident that in the majority of cases, streptococci act as the infective agents. Occasional outbreaks or series of cases occur in which some other types of organism may predominate for a time, but these are exceptional. For instance, after the first case of mastitis due to Bacillus coli infection appeared on farm 3, five of the next nine cases were also due to that organism but were scattered over a period of six months so that no direct connection between the cases can be claimed. It is apparent, however, that in this barn there was a source of infection which was disseminating virulent colon bacilli pathogenic for the udders of cattle.

The reports by Jones [3] constitute one of the most complete studies of the bacteriology of bovine mastitis that has appeared in recent years. His reports include a review of the literature, a phase of the subject which has not been included in this paper. We have prepared a compilation of cases which shows the principal organisms isolated from mastitis cases by a number of investigators. The significance of the various organisms as infective agents in bovine mastitis is apparent from this compilation and requires no comment (table 3).

A number of workers have studied the experimental production of mastitis in cows, using a variety of organisms. The usual mode of infection employed was injection of the material into the teat canals, which is the most likely path of infection in spontaneous cases except in tuberculous mastitis and similar conditions. Mathers [4] employed hemolytic streptococci of human origin and produced severe inflammation of

[3] J. Exper. Med., 1918, 18, pp. 149, 253, 721, 735.
[4] J. Infect. Dis., 1916, 19, p. 222.

the udder which led, in some cases, to a chronic inflammatory condition with resultant atrophy of the mammary gland. On the other hand, Mathers, as well as others, has pointed out that virulent hemolytic streptococci and other organisms may grow and multiply in the milk ducts of cows without causing any visible changes in the udder; however, the presence of pathogenic organisms, streptococci or others, and an increased number of leukocytes in milk are indicative of mastitis even though clinical signs of the disease are lacking.

Carpenter [5] found that milk containing hemolytic and nonhemolytic streptococci from infected udders produced more severe mastitis than 24-hour broth cultures of the same organisms when equal amounts were

TABLE 3

Types of Organisms Isolated in Cases of Mastitis: Summary

Investigator	Cases	Strep-to-cocci	Staph-ylo-cocci	Colon Group	Bacillus lactis aërog-enes	Ba-cillus pyog-enes	Ba-cillus tuber-culosis	"Ga-lac-to-cocci"	Mixed Infec-tion	Not Iden-tified
Lucet*............................	21	2	..	..	..	..	..	..	..	19
Guillebeau*.......................	85	11	33	22	..	..	..	19	..	..
Steiger*..........................	45	10	6	14	..	..	..	10	6	..
Savage*..........................	31	21	5	1	..	..	1	..	..	3
Henderson*.......................	14	..	..	..	..	..	2	..	12†	..
Zwick and Weichel*...............	21	..	..	2	19	..	..	..	..	..
Jones............................	81	50	..	2	1	..	..	24‡	..	4
Carpenter........................	150	128	9	3	..	6	..	..	1	2
Hardenbergh and Schlotthauer..	68	40	7	6	1	..	..	..	8	1
Total........................	516	262	60	50	21	6	3	53	27	29
Cases due to each organism, %........		50.7	11.4	9.7	4.0	1.1	0.58	10.2	5.2	5.6

* Quoted by Jones.[3]
† Bacillus coli and streptococci.
‡ Classified as micrococcus by Jones, many strains being staphylococcus.

injected into the teat canals of healthy cows. Streptococcus viridans isolated from diseased genital organs, Bacillus abortus and Bacillus pyocyaneus produced only slight inflammation of the udder when injected, whereas Bacillus coli produced acute mastitis that cleared up eight days after injection. Carpenter also found that Staphylococcus aureus and Pasteurella boviseptica produced severe mastitis which destroyed the functional activity of the gland. He also believed that the age of the animal and the amount of milk given by the animal at the time of injection are two factors which influence the degree of mastitis produced.

SUMMARY

The results of the study of 68 cases of mastitis indicate the prevalence of streptococci as infecting agents in the disease, although other organisms, particularly staphylococci and members of the colon-typhoid group

[5] J. Infect. Dis., 1922, 31, p. 1; J. Am. Vet. M. A., 1925, 67, p. 317.

may show occasional periods of predominance. The veterinarian seldom sees cases of bovine mastitis not complicated by infection. It is believed that the incidence of the more severe cases of mastitis can be materially reduced in all herds by adherence to a few simple rules of dairy hygiene, such as the isolation of mastitis cases, avoidance of disseminating the infection from infected cases, prevention of dietary disorders, and precautions against traumatism.

EXCRETION OF ANTISEPTIC DYES THROUGH THE MAMMARY GLAND

VICTOR BURKE AND E. A. RODIER

From the Bacteriological Laboratories of the State College of Washington

The occurrence of infectious abortion in dairy herds has until recently been considered mainly as an economic problem. The economic loss to the cattle industry has been enormous. Recent reports on the occurrence of Brucella abortus and Br. abortus, var. melitensis, infections in man suggest that infectious abortion in cattle may be of vastly greater hygienic importance than heretofore recognized.[1] The hygienic aspects of this organism may become far more important than the economic ones. The organism frequently lodges in the udder of dairy cattle and the milk from such animals offers an uninterrupted source of infection to those using such milk. The causal relationship between infectious sterility in women and milk supplies contaminated with Br. abortus remains to be established. Recent reports indicate such a possibility. If the occurrence of Br. abortus in milk proves to be a serious menace to the health and reproduction of man, the dairy industry must face the necessity of ridding the herds of the organism or pasteurizing the milk.

An intravenous antiseptic that is secreted through the mammary gland should prove of value in ridding herds of Br. abortus and checking udder infection such as mastitis. Such an antiseptic may prove of value in human medicine since the female breast is occasionally a source of infection to the nursing child. The present article describes experiments made to determine whether the antiseptic dyes are excreted through the mammary gland.

Three antiseptic dyes, gentian violet, acriflavine and mercurochrome-220 are excreted unaltered by the kidney following intravenous injection. The urine has bacteriostatic action. This action is greater if the urine is alkaline. Reports indicate that injection of the dyes may be beneficial in some cases of septicemia, and in infections of the kidney and bladder. Some case reports are unfavorable. The fact that various foreign substances are excreted by the mammary gland led us to determine whether

[1] Evans, Alice C.: Pub. Health Rep., 1924, 39, p. 501; (Editorial) Human Infections with Bacillus Abortus, J. A. M. A., 1925, 84, p. 1047; Carpenter, C. M., and Merriam, H. E.: Ibid., 1926, 87, p. 1269; Gage, E. E., and Gregory, D. A.: Ibid., p. 848.

the antiseptic dyes would also be excreted and in sufficient amount to inhibit bacterial growth in the udder.

Exper. 1.—To determine the effect of an intravenous injection of neutral acriflavine on the bacteriostatic action of milk.

A jersey cow giving three gallons of milk per day was selected for the experiment. Counts on successive days of the bacteria in the milk as drawn gave an average of 40 organisms per cc. Fifteen minutes before the regular milking time the cow was given an injection in the jugular vein with 7 mg. per kilo of neutral acriflavine (Abbots) in buffered physiologic sodium chloride solution (0.85 gm. sodium chloride, 0.300 gm. of potassium dihydrogen phosphate and 0.387 gm. dipotassium hydrogen phosphate to 100 cc. distilled water). The solution was injected at body temperature over a period of 12 minutes. Physiologic salt solution preceded and following the dye through the needle into the vein. This was to avoid seepage of the dye into the subcutaneous tissue where it causes a severe edema. The milk when drawn at milking time was highly colored with acriflavine. The strippings appeared to contain the greatest concentration of the dye.

Samples of milk taken at the time of the injection and of the fore milk, middle milk and strippings were plated out immediately and at 4 and 16 hours. The plates failed to indicate an appreciable difference in rate of multiplication of the organism in the 4 samples. Similar samples were inoculated with Bact. coli and Staphylococcus albus and plated out as above. Similar results were obtained. Similar samples were placed in the incubator and rate of fermentation noted. No difference was noted in the rate of fermentation of the control and the three tubes containing the dye colored milk. Samples of milk were drawn each hour up to 4 hours after milking. The milk showed no trace of dye at the 4th hour.

The experiment was repeated 9 days later using 10 mg. of neutral acriflavine per kilo of body weight. Similar results were obtained.

The experiment was repeated with a different cow with the exception that 11 mg. per kilo of neutral acriflavine in physiologic saline was injected. There was severe diarrhea, the milk flow was retarded for several hours and the cow refused to eat. The volume of milk was reduced from 2 gallons per milking to 1½ pints. This was well colored with the dye. Experiments failed to demonstrate greater bacteriostatic action in this milk than in the control milk. The cow's condition appeared normal at the next milking period.

The results demonstrate that neutral acriflavine is excreted through the mammary gland in sufficient amount to color the milk. The experiment failed to demonstrate increased bacteriostatic action in such milk.

Exper. 2.—To determine the effect of intravenous injections of mercurochrome-220 on the bacteriostatic action of milk.

The cow used in experiment 1 was given an injection with 5 mg. per kilo of mercurochrome-220 in a 1% solution in distilled water. The injection was made 15 minutes before the regular milking time. The cow showed no symptoms at the time of injection. The color and bacteriostatic action of the milk was unaltered. Apparently this dye in the dosage used is not excreted through the mammary gland.

Twenty hours after the injection of the dye the cow developed severe symptoms as evidenced by diarrhea with cherry red feces, muscular tremors, salivation, suppression of urine, rough coat, weakness, loss of appetite and reduction of milk flow. Certain of the symptoms persisted for weeks. The milk flow was greatly reduced and the animal was sold.

Exper. 3.—To determine the effect of an intravenous injection of gentian violet on the bacteriostatic action of milk.

The only cow available for this experiment was one near the end of the lactation period and giving about a quart of milk night and morning. This animal, because of the little flow of milk, was not a satisfactory object for our purpose. Nevertheless the experiment was carried out.

It was planned to give the animal 5 mg. per kilo of a 1% solution in distilled water.

The animal showed severe symptoms of shock and prostration when about 3 mg. per kilo had been injected and the injection was discontinued. No dye appeared in the milk.

In an effort to find a means of avoiding shock to the animal the experiment was repeated twice with the dye prepared in buffered physiologic sodium chloride. Sodium chloride causes rapid decomposition of gentian violet solutions.[2] It should be added just before the injection. The value of such solutions is doubtful. Following both injections the cow showed severe reactions and no dye appeared in the milk. Apparently the buffering and addition of sodium chloride did not reduce the toxic action of the dye solution.

The results obtained in experiment 3 concerning the excretion of gentian violet through the mammary gland are inconclusive and should be repeated on an animal giving a heavy flow of milk.

DISCUSSION

Our experiments have demonstrated that at least one of the antiseptic dyes, neutral acriflavine, is excreted by the mammary gland apparently unaltered. The experiments failed to demonstrate that the dye is excreted in sufficient amount to increase the bacteriostatic action of the milk. Failure to demonstrate an increase in the bacteriostatic action of the milk should not be considered as conclusive evidence that the dye would not be beneficial in checking bacterial multiplication in the udder. The injection of dyes in the blood stream in insufficient amounts to appreciably increase the bacteriostatic action of the blood has proven of value in septicemia and infections of the urinary tract. The dye in the udder can be made to act over a longer period of time than in the blood stream. Dyes in the blood stream are excreted in an hour or two. Dyes can be left in the udder for a longer period. The value of dye therapy in the treatment of infections of the mammary gland remains to be

[2] Burke, Victor; and Newton, J. L.: J. Am. M. A., 1926, 86, p. 529.

determined. It is quite possible that with the development of the proper technic it will be found possible to successfully treat infections of the udder by intravenous injection and local application of dyes. The possibility of ridding herds of Brucella abortus by this method should stimulate investigations.

CONCLUSIONS

Neutral acriflavine was excreted through the mammary gland of the cow following the intravenous injection of from 7 mg. to 11 mg. per kilo. The milk was well colored. The injection of 11 mg. per kilo caused a severe reaction and a temporary cessation of the milk flow. Although the experiments failed to demonstrate an increase in the bacteriostatic action of the milk following the intravenous injection of neutral acriflavine there is reason for believing that injection of the dye may affect the organism in the udder.

The intravenous injection of gentian violet caused more severe reactions than acriflavine, and mercurochrome caused still more severe and prolonged symptoms. Apparently these dyes were not excreted by the mammary gland.

THE BACTERIOLOGY AND CHEMISTRY OF ADULT DUODENAL CONTENTS

STUDIES IN BACTERIAL METABOLISM. LXXXII

ARTHUR ISAAC KENDALL, ALEXANDER ALFRED DAY, ARTHUR WILLIAM WALKER,
AND REBA CORDELIA HANER

*From the Department of Bacteriology and Public Health, Washington University School of
Medicine, St. Louis, Mo., and the Department of Bacteriology, Northwestern
University Medical School, Chicago*

The duodenal tract of a normal individual is an important part of the alimentary canal, not only because it receives secretions from neighboring glandular organs, but also because it is the place of initial development of that vast column of bacteria, numbering some thirty trillions, which is excreted daily in the fecal mass.

It is rather generally held that the normal flora of the duodenum, both alive and dead, is surprisingly limited in number and restricted in kind. Even in diseased conditions the microbic population is not believed to be greatly augmented except in cases of intestinal obstruction or marked stasis. Achlorhydria however, with or without gastric or duodenal neoplasm, tends to enhance the growth of bacteria in the duodenal area. This is assumed to be due largely to the absence of free hydrochloric acid in the gastric juice. Readily ionizable acids are potent sterilizing agents.

Lactic acid is generated in the stomach during digestive periods in cases of achlorhydria, and the microbic flora therein may be quite extensive. In these cases, the bacteria are derived partly from the normal residents of the mouth and partly from the organisms in the food, some of which find conditions in the achlorhydric stomach favorable for their development. The duodenum may receive thereby large numbers of viable bacteria.

From the viewpoint of nutrition, the duodenum should be, theoretically at least, an advantageous microbe incubator. The partially hydrolized fragments of food protein together with carbohydrates, fats, salts and water, and a uniform optimum temperature would appear to offer a most favorable environment for bacterial growth. Yet most observers, as Escherich,[1] Köhlbrugge,[2] Hoefert,[3] Rolly and Libermeister,[4] Ganter and Van der Reis,[5] merely to mention a few of those who have made extensive studies of the duodenal flora, have found relatively few or even no bacteria capable of growing in ordinary cultural mediums at this level.

[1] Die Darmbakterien, 1886.
[2] Centralbl. f. Bakteriol., I, O., 1901, 29, p. 571; 30, p. 10.
[3] Ztschr. f. klin. Med., 1921, 92, p. 221.
[4] Deutsch. Arch. f. klin. Med., 1905, 83, p. 413.
[5] Ibid., 1921, 137, p. 348.

Indeed, several investigators have reported experiments which seem to point to an actual destruction of microbes artificially implanted in the duodenum. This viewpoint culminated in the "Bacteriostanne" theory of Bogendörfer,[6] a theory that would endow the mucosa of this part of the intestinal tract with microbicidal properties. This work has been examined carefully by Radel,[7] and not corroborated.

The introduction of the duodenal tube has made the study of the duodenal flora much more feasible as a routine than formerly, and the work of MacNeal and Chace,[5] Hoefert,[3] Gorke,[9] and Goldman,[10] indicates quite clearly that the duodenum does harbor moderate numbers of bacteria among which are, or may be, many types.

Prominent among these types are Enterococci (Escherich,[1] Kendall and Haner[11]), lactic acid forming organisms, both gram-positive and gram-negative, and members of the Mucosus capsulatus group, particularly the starch fermenting types.[12] Adventitious forms, including members of the mesentericus group, are quite commonly encountered. It should be remembered, however, that the latter organisms grow rapidly in the ordinary cultural mediums, whereas the more strictly parasitized types, as the enterococci, grow less luxuriantly, especially in primary inoculations outside the body.

The duodenal tube, controlled by fluoroscopic examination, is a reliable instrument for withdrawing material from the upper intestinal tract but it must be borne in mind that it is difficult, indeed well near impossible, to prevent contamination with mouth organisms, even though the orifice of the tube be sealed with gelatin or other substance to reduce this contingency to a minimum. Nevertheless, the results are reasonably satisfactory if the patient be induced to brush the teeth carefully, and to use some suitable mildly antiseptic gargle prior to swallowing the tube. It can be withdrawn rapidly. The comparative rarity of mouth organisms in cultivations from duodenal contents, thus obtained would seem to bear out this contention; the occasional bacteria free or nearly sterile sample is also reassuring.

From the viewpoint of diet, the proximity of the duodenum to the stomach would postulate a continuous, or nearly continuous admixture of carbohydrate in the food during digestive periods. This might be expected to be reflected in a predominance of fermenting types of bacteria over proteolytic types at this level. The catalogue of frequently occurring types enumerated previously is in harmony with this hypothesis. It should be borne in mind in this connection that many, if

[6] Ztschr. f. d. Ges. exper. Med., 1924, 41, p. 637.

[7] Ibid., 1926, 48, p. 658.

[8] Arch. f. Int. Med., 1913, 12, p. 178.

[9] Mitt. a. d. Grenz geb. d. Med. u. Chir., 1922, 35, p. 279.

[10] J. Infect. Dis., 1924, 34, p. 459.

[11] J. Infect. Dis., 1924, 35, p. 67.

[12] Bogendörfer: Deutsch. Arch. f. Klin. Med., 1922, 140, p. 257.

not a majority, of the intestinal bacteria in adolescents and adults are facultative with respect to their fermentative powers, however, and a temporary paucity of carborhydrate would merely force the normal intestinal organisms to derive their energy requirements from protein and protein derivatives pending a more natural balance in the diet between these two great classes of food stuffs. Under such conditions the types of bacteria should not undergo any noteworthy change in appearance. The nature of the products of metabolism would be very different, however. Thus a colon bacillus produces lactic acid, small amounts of formic acid, carbon dioxide and hydrogen upon a carbohydrate diet as its principal products of metabolism, whereas indol, ammonia and other substances indicative of the metabolism of proteins, are commonly encountered when the microbe is forced to utilize this type of food for energy.[13]

In seeking for an explanation of the relative paucity of the duodenal flora in health and under otherwise natural conditions, it is essential to bear in mind the generally accepted theory of the disinfectant action of the normal gastric juice on the one hand, and the ebb and flow of the food through the duodenum on the other hand. These two factors alone would seem to explain plausibly the more striking features of normal duodenal bacteriology without invoking a specific "Bacteriostanine" factor, or other quasi vitalistic mechanism. Also the experience derived from surgical operations upon the upper intestinal tract, suggests that this region is a rather less hazardous site for infection than stretches of the alimentary canal lower down. Nevertheless, available evidence, however satisfying in the light of current information, must not be accepted as proof. There is still fruitful field for the study of bacterial survival in the duodenum.

The observations presented below are based upon a study of the duodenal contents of 50 adults, none of whom presented noteworthy symptoms indicative of duodenal disease. The material was obtained through the courtesy of Dr. Frank Wright of Chicago, to whom we are indebted, not only for the specimens, but also for the careful manner in which they were taken.

Procedure.—The material was planted within two hours in the usual cultural mediums—plain and sugar broths, and in milk, using 1 cc. of duodenal contents, thoroughly mixed for each 100 cc. of culture. Incubation was practiced in Erlenmeyer flasks of 250 cc. capacity, and in the anerobic flasks described

[13] Kendall: Physiological Reviews, 1923, 3, p. 438 (for details).

previously.[14] The latter were freed from oxygen immediately before inoculation. It will be seen therefore that duplicate mediums were employed, the one freely exposed to the air, the other protected from the access of air. Microscopic examination of the duodenal content, and the contents of the various flasks were made, each at the proper time. Chemical examinations[15] were made of the several mediums at the end of 1, 3, 6 or 7, and 9 day intervals. Separate flasks of the several mediums were used for each period. Pure cultures of the predominating types of bacteria were isolated at these respective intervals, and the predominating types were compared with the chemical findings so far as this was possible.

DISCUSSION

Twenty-six samples, or 52% of all the series, showed little or no growth. The direct examination of the duodenal material in each of these samples by Gram's method showed practically no bacteria. Well marked gas formation in the dextrose, lactose, saccharose and starch fermentation mediums was observed in 14 samples, or 28% of the total. Milk was coagulated, but not peptonized in each of these specimens, and there was relatively little change in the nitrogenous constituents of the plain broth and gelatin mediums. Five specimens showed some gas in dextrose, lactose, and usually saccharose, but practically none in the starch medium. The milk coagulated, usually within 72 hours of incubation and became almost completely peptonized by the sixth or seventh day. Spore forming bacteria of the mesentericus type were present in relatively large numbers in each of these cases. However, as will appear later, such spore forming aerobic bacteria were obtained from seven other specimens, although in smaller numbers. The Welch bacillus (Bacillus welchii) was obtained in abundance in three of the specimens, or in 6% of the total number, and Bacillus pyocyaneus grew luxuriantly in all the cultures from one sample.

It will be seen that rather more than half of these duodenal contents contained so few bacteria (or possibly, such fastidious organisms), they failed to show more than the faintest growth, even after nine days' incubation. This percentage is so great, it must be regarded as corroborative of the experience of other observers that the duodenal flora may be very limited in many instances. It would have been illuminating to obtain several successive specimens from one person exhibiting this amicrobic condition for comparison. It should be stated in this connection that all the persons studied partook of no food for at least twelve hours before the duodenal tube was passed.

[14] Kendall, Cook and Ryan: J. Infec. Dis., 1921, 29, p. 227.
[15] Kendall: Ibid., 1922, 30, p. 211 (for procedure).

The following tabulation shows the frequence with which several of the more conspicuous bacilli were identified:

Organism	Times isolated	Percent of cases
Mucosus capsulatus group (starch fermenting only)....	14	28
Micrococcus ovalis....................................	15	30
Staphylococcus	20	40
Streptococcus	8	16
Bacillus coli group (9, or 18% were B. acidi lactici)...	14	28
Mesentericus group (aerobic, sporeforming, gelatin-liquifying)	12	24
Bacillus pyogenes foetidus	10	20
Yeast	7	14
Bacillus pyocyaneus	1	2
Bacillus welchii	3	6
Bacillus acidophilus	7	14
Alcaligenes group	6	12
Sarcinae	2	4

It is somewhat surprising to find staphylococci so numerous; several of these were tested for hemolytic power upon blood agar and 4 out of 10 were found to digest hemoglobin,[16] leaving a clear halo around individual colonies. Micrococcus ovalis was also commonly found in those specimens from which significant growth was obtained. The organism, however, is usually regarded as a normal inhabitant of the duodenum. None of the strains encountered was hemolytic.[11] Starch fermenting members of the Mucosus capsulatus group were isolated from each of the 14 specimens which yielded considerable amounts of gas in the starch fermentation medium. The colonies on saccharose agar plates were distinctly viscid, and there was a fairly characteristic "sour" odor to such plates, provided the colonies were quite abundant. Of the remaining organisms, two are noteworthy. One, the alkaligenes-like bacillus, is certainly not the typical organism of that name; the several strains isolated produced much deamination in plain broth mediums—amounting to some 67 mg. per 100 cc. of broth culture, and 87 mg. in gelatin cultures after seven days' incubation. Judging from the very large amounts of ammonia produced in mixed cultures where these organisms were prominent—some 110 mg. in plain broth, and 156 mg. in gelatin in seven days in one instance—the microbe is an important member of the nonsporeforming, protein digesting bacteria of the alimentary tract. In many of its characteristics it suggests the "ammonium bacillus" of Cook,[17] but it is gram-negative in its staining

[16] Van Loghem: Centralbl. f. Bakteriol., I, O., 1912, 67, p. 410.
[17] Am. J. Dis. Child., 1921, 22, p. 481.

reaction whereas Cook's organism retains the Gram stain. There are other points of differentiation as well.

The other organism which is of significance is the "gas bacillus," which was very abundant in three of the specimens. It will be recalled that the gas bacillus group occurs in moderate numbers in a majority of fecal specimens obtained from normal, healthy adults. The isolation of this organism from duodenal contents is not often reported, nor does the microbe occur commonly at this level of the intestinal tract.

Ordinarily, gas bacilli are obtained most readily from milk cultures, heated to 80 C. after inoculation, and prior to incubation. This precaution is required to eliminate nonspore forming lactic acid producing bacteria, which otherwise crowd out the gas bacilli before they have a chance to vegetate and get a start. In the three positive specimens herein reported, the milk was not thus heated after inoculation: nevertheless, the organism outgrew the associated bacteria and induced in the milk the well known "stormy fermentation." This seems to point both to an abundance of gas bacilli and a reduction in the numbers of lactic acid formers. It is possibly of significance that the alcaligenes-like bacilli discussed above were very abundant in these three specimens.

It might be argued that gas bacilli were present, but not identified, in other duodenal specimens. As a matter of fact, all the specimens studied were tested for gas bacilli by the method of heating to 80 C. prior to incubation, and with three exceptions found to be negative. These three contained enough gas bacilli to induce the "stormy fermentation" both in the heated (80 C.) and the unheated milk.

The possible significance of gas bacilli in the upper intestinal tract has been discussed elsewhere.[18] It will be recalled that the organism produces not only a hemolysin, a soluble poison, and butyric acid in considerable amounts, but also a histamine-like substance that induces both prolonged contracture in isolated guinea-pig intestine and uterus, and also a well marked but transient lowering of blood pressure in the dog under ether anesthesia.[19]

The chemical changes induced in cultural mediums by the mixed duodenal flora are set forth in the table below. It will be recalled that 26 of the 50 specimens of duodenal contents showed little or no growth in artificial mediums. The remaining 24 specimens are classified, for convenience, into seven groups, based upon the amount of ammonia developed in plain sugar free broth mediums at the end of seven days

[18] Kendall: J. Am. M. A., 1926, 86, p. 737.
[19] Kendall and Schmitt: J. Infect. Dis., 1926, 39, p. 250.

incubation. This classification is purely arbitrary, although, as will be shown in tabular form, the several groups form an ascending series of proteolytic gradients, which has some relationship to the relative abundance of putrefactive organisms. The division into groups on the basis of the milligrams of ammonia per 100 cc. of plain broth culture medium is as follows:

Group	Mg. Ammonia	Number of specimens
1	0 to 10	5
2	11 to 20	3
3	21 to 30	2
4	31 to 40	2
5	41 to 60	6
6	61 to 100	5
7	101 to —	1

It would be purposeless to repeat here each individual, complete chemical analysis, but an average taken from each group is tabulated (table 1) to indicate the general trend of the metabolism of a specimen duodenal contents of this type.

The most conspicuous feature of the metabolism of the combined duodenal floras shown in the table, aside from their progressive proteolytic capacities, which is of course postulated by the grouping, as explained previously, is the sparing action which the several carbohydrates exhibit for the protein constituents of the cultural mediums. The significance of this sparing action of utilizable carbohydrate for protein has been commented upon in several earlier communications.[13] One aspect of this sparing action deserves special comment in association with the data presented above, namely, that the types of bacteria encountered in protein mediums, and protein-carbohydrate mediums respectively, do not as a rule show violent fluctuations in type distribution; that is to say, the alteration of the cultural constituents utilizable for energy is not reflected in a complete separation of the duodenal microbes into those that grow in purely protein mediums and those that develop only in carbohydrate-containing mediums. Rather, the organisms encountered, with the rather conspicuous exception of gas bacilli, Micrococcus ovalis, and members of the Bacillus acidophilus groups, grow with considerable luxuriance in both kinds of mediums. This suggests that temporary paucity of carbohydrate does not cause either an immediate change in, or an abrupt unbalancing of, the microbe flora of the duodenum. Continued deprivation of carbohydrate, however, should eventually bring about conspicuous changes in the duodenal microbic types.

TABLE 1

The Metabolism of the Duodenal Flora, Arranged According to Groups

Analyses in mg. per 100 cc. culture medium. Reaction in cc. of normal acid (+) or alkali (—) per 100 cc. culture medium.

Group	Aerobiosis	Plain Reaction	Plain Am-monia	Plain Amino Nitrogen	Glucose Reaction	Glucose Am-monia	Glucose Amino Nitrogen	Saccharose Reaction	Saccharose Am-monia	Saccharose Amino Nitrogen	Starch Reaction	Starch Am-monia	Starch Amino Nitrogen	Gelatin Reaction	Gelatin Am-monia	Gelatin Amino Nitrogen	Milk Reaction	Milk Am-monia	Milk Amino Nitrogen
1	Aerobic	+0.30	6.3	6.3	+3.60	5.6	7.0	+3.50	4.9	6.3	+2.50	1.4	4.2	+0.50	6.3	38.5	+4.30	3.5	13.3
	Anaerobic	+0.10	9.1	9.8	+2.50	2.1	4.9	+2.70	4.2	4.2	+2.90	6.3	3.5	−0.40	14.7	38.5	+15.1	7.7	14.7
2	Aerobic	+0.20	15.4	7.0	+2.80	1.4	4.2	+3.00	4.2	3.5	+2.80	5.6	4.2	+0.70	36.4	46.2	+5.90	3.5	3.5
	Anaerobic	+0.20	7.0	4.2	+2.60	0.7	2.1	+2.90	2.1	1.4	+3.30	2.8	1.4	+0.50	32.9	34.3	+5.70	5.6	2.8
3	Aerobic	−1.10	26.6	8.4	+1.80	2.1	7.7	+1.90	4.9	5.6	+1.40	4.9	8.4	−2.40	91.7	122.5	+4.00	3.5	2.8
	Anaerobic	−0.30	11.9	4.9	+0.90	0	4.2	+0.60	4.2	3.5	+1.50	4.2	6.3	+0.40	63.7	49.0	+4.70	4.2	2.1
4	Aerobic	−1.80	35.0	5.6	+3.60	0.7	0	+3.60	0.7	2.1	+3.80	0.7	0.7	−2.10	79.1	29.4	+6.70	1.4	4.2
	Anaerobic	+0.60	46.2	1.4	+3.00	0.7	2.1	+3.70	0	0	+3.10	2.1	3.5	+1.00	109.9	29.4	+9.20	2.8	3.5
5	Aerobic	−1.20	52.5	14.7	+2.20	7.0	8.4	+2.40	5.6	8.4	+2.50	6.3	7.7	−3.10	149.8	42.0	+6.90	7.0	4.9
	Anaerobic	+0.40	22.4	5.6	+1.30	4.9	4.2	+2.20	2.8	5.6	+2.40	3.5	6.3	+0.60	182.0	37.8	+4.80	2.1	2.1
6	Aerobic	−2.70	99.4	18.2	+2.90	9.8	7.0	+3.00	7.7	10.5	+3.00	9.8	10.5	−3.30	183.4	93.1	+4.50	4.9	3.5
	Anaerobic	+0.30	79.8	9.8	+1.60	3.5	6.3	+2.10	2.1	7.7	+2.20	5.6	9.8	+1.40	147.0	49.0	+5.20	2.8	2.8
7	Aerobic	−2.30	110.6	28.0	+1.10	9.1	4.9	+1.20	8.4	5.6	+1.20	2.8	4.2	−2.30	130.2	177.8	+3.80	11.9	9.8
	Anaerobic	+0.80	67.9	9.1	+1.20	1.4	4.2	+0.90	2.1	2.1	+1.60	9.8	1.4	+2.70	156.1	25.9	+4.60	2.1	7.7

On the other hand, the products produced by many of these facultative microbes would be very different in the several mediums. As a general rule such substances, as indol and skatol, indicative of the utilization of protein derivatives for energy, are produced in considerable amounts by the mixed flora developing in mediums free from carbohydrates; the same flora produces much lactic acid together with formic and probably some acetic acid in corresponding mediums to which carbohydrate is added. The Enterococcus (Micrococcus ovalis) and members of the acidophilus-bifidus group do not thrive in the absence of carbohydrates and therefore are poorly represented in sugar free mediums.

The gas bacillus (Bacillus welchii) is unlike most of the other more or less normal intestinal inhabitants both in that its growth is much restrained by the simultaneous growth of normal intestinal bacteria in mediums where lactic acid is formed freely, and also in the character of its significant products of growth. Upon mediums containing but little or no carbohydrate, a soluble poison [20] (for which an antitoxin may be prepared [21]) and quite frequently a hemolysin as well [22] is produced. The addition of carbohydrate reduces materially the formation of poison and of hemolysin, but induces the production of much butyric acid,[23] and especially of a histamine-like substance, in relatively considerable amount.[19, 24] It is significant that this histamine-like substance, though probably formed from amino acid complexes containing histidine requires the stimulation of the carbohydrate to elaborate the carboxylase, which in turn removes the carboxyl group from the amino acid containing the aromatic nucleus, leaving behind the physiologically reactive ethyl amine derivative. It is of significance that none of these several products of the gas bacillus, except possibly butyric acid, appears to be absorbed as such, through the normal intact intestine mucosa.

A partial study of the nitrogenous metabolism of freshly isolated cultures of several of the more commonly encountered duodenal bacteria was also made, both in aerobic and in anaerobic cultures. The anaerobic cultivations were introduced not only because comparative figures for the two conditions do not seem to have been made previously, but also, because it was surmised that anaerobic conditions usually prevail in the duodenum.

[20] Bull and Pritchett: J. Exper. Med., 1917, 26, p. 119.
[21] Bull: Ibid., p. 603.
[22] Herter: J. Biol. Chem., 1906, 2, p. 1; Simonds, Monograph 5. Rockefeller Inst. for Med. Research, 1915.
[23] McCampbell: J. Infect. Dis., 1909, 6, p. 537.
[24] Kendall and Schmitt: Proc. Soc. Exper. Biol. & Med., 1926, 24, p. 104.

TABLE 2

The Metabolism of Some of the Organisms Isolated from Duodenal Contents

Analyses in milligrams per 100 cc. medium: Reaction in cc. of normal acid (+) or normal alkali (−) per 100 cc. culture medium

Organism	Days of Incubation	Plain Reaction	Plain Ammonia	Plain Amino-Nitrogen	Glucose Reaction	Glucose Ammonia	Glucose Amino-Nitrogen	Saccharose Reaction	Saccharose Ammonia	Saccharose Amino-Nitrogen	Starch Reaction	Starch Ammonia	Starch Amino-Nitrogen	Gelatin Reaction	Gelatin Ammonia	Gelatin Amino-Nitrogen	Milk Reaction	Milk Ammonia	Milk Amino-Nitrogen
M. ovalis Aerobic	2	+0.10	5.6	+5.6	+1.80	4.2	+4.2	+1.10	7.0	+2.8	+0.10	6.3	+3.5	+0.30	6.3	+0.7	+2.20	0	+2.8
	7	−0.10	7.0	+9.8	+2.70	4.2	+4.2	+1.70	7.0	+4.2	+0.50	7.0	+8.4	+0.60	6.3	+11.9	+4.90	0.7	+6.3
Anaerobic	2	0.00	6.3	+2.8	+1.20	4.2	+2.8	+0.50	5.6	+1.4	+0.00	6.3	0.0	+0.10	6.3	0.0	+0.60	0.7	+2.1
	7	0.00	7.0	+2.8	+3.00	5.6	+4.2	+1.70	7.0	+4.2	0.00	7.7	+2.1	+0.50	8.4	+7.0	+5.40	0.7	+6.3
B. pyocyaneus Aerobic	2	−0.20	16.8	−9.8	+0.80	12.6	−8.4	0.00	12.6	−8.4	−0.40	18.9	−12.6	−0.40	23.8	−15.4	−0.70	0.0	0.0
	7	−1.10	28.0	−14.0	+1.20	23.8	−15.4	−0.60	23.8	−15.4	−0.70	21.7	−12.6	−0.80	23.8	−12.6	−1.10	2.1	−0.7
Anaerobic	2	0.00	2.1	−2.1	0.00	4.2	−2.8	0.00	2.8	−2.8	0.00	2.8	−2.8	−0.10	1.4	−1.4	+0.10	2.1	−0.7
	7	−0.10	7.0	−4.2	0.00	4.2	−4.2	0.00	3.5	−2.1	0.00	2.8	−2.8	−0.10	6.3	+2.1	+0.10	2.1	−0.7
Number 62* Aerobic	2	−0.10	19.6	+2.8	0.00	15.4	+4.2	−0.10	16.8	+5.6	−0.20	17.5	+3.5	−0.40	26.6	+25.2	−0.10	13.3	+4.9
	7	−2.10	67.2	−5.6	−0.90	39.9	−0.7	−0.90	47.6	−0.7	−2.50	56.0	−1.4	−2.90	87.5	+192.5	−0.50	55.3	+28.7
Anaerobic	2	0.00	1.4	−1.4	0.00	0.7	−0.7	0.00	0.7	−0.7	0.00	1.4	−2.8	−0.10	0.7	−1.4	0.00	0.0	0.0
	7	−0.40	2.8	+5.6	+0.10	2.1	+9.1	0.00	2.8	+7.0	0.00	4.2	+5.6	0.00	3.5	+3.5	0.00	0.0	0.0
B. lactic aerogenes† Aerobic	2	+0.10	3.5	−0.7	+0.70	0.7	+0.7	+0.70	0.7	−0.7	+0.20	1.4	−0.7	+0.10	1.4	−1.4	+1.40	0.0	0.0
	7	−1.60	32.9	−12.6	−1.20	23.8	−11.2	−1.30	23.8	−11.2	−1.30	27.3	−13.3	−1.10	17.5	−8.4	+3.50	0.0	+1.4
Anaerobic	2	+0.20	1.4	0.0	+0.80	0.7	+0.7	+0.90	0.0	+0.7	+0.40	0.7	−0.7	−0.10	0.7	−2.1	+1.60	0.0	0.0
	7	−0.10	4.9	+1.4	+0.80	0.0	+6.0	+1.30	0.7	+0.7	+0.80	2.1	−0.7	−0.20	2.1	−2.1	+2.30	0.7	+2.1
B. coli Aerobic	2	+0.10	2.1	−0.7	+1.60	0.7	−0.7	+1.50	1.4	0.0	+0.20	2.8	−1.4	+0.40	1.4	0.0	+1.40	2.1	−0.7
	7	−1.40	25.9	−13.3	+1.70	0.7	−2.1	+1.60	1.4	−1.4	−1.20	23.8	−12.6	−0.60	13.3	−10.5	+3.40	0.0	0.0
Anaerobic	2	+0.30	2.1	−2.1	+1.50	1.4	0.0	+0.30	2.1	−0.7	+0.20	2.1	−0.7	+0.20	2.1	−0.7	+0.60	2.1	−2.1
	7	−0.10	3.5	−2.1	+1.10	0.7	−2.1	+1.20	0.7	−0.7	+0.60	3.5	−2.1	0.00	3.5	−4.9	+2.30	2.1	−1.4

* Alcaligenes-like proteolytic bacillus.

† Organism ferments all ordinary carbohydrates including starch.

These organisms comprised Micrococcus ovalis, Bacillus pyocyaneus, Bacillus lactis aerogenes, Bacillus coli and a bacillus, number 62, which is unable to ferment any sugars, in which respect it agrees with Bacillus alcaligenes; but, unlike the latter it produces rather profound changes in the protein constituents of the mediums principally through the production of much ammonia. The ammonia produced in broth cultures amounted to as much as 67 mg. per 100 cc., and nearly 88 milligrams in the gelatin broth medium by the seventh day of incubation. The reaction naturally was quite alkaline at that time. The association of this organism with the gas bacillus takes on significance in the light of the intolerance of the latter for an acid environment. In the mediums from which oxygen was excluded, this alcaligenes-like bacillus grew very feebly, and it is not at all clear therefore why this microbe was so abundant in 6 of the duodenal specimens.

The lactis-aerogenes culture is noteworthy for the fact that in all mediums containing carbohydrate, milk being a notable exception, the reaction was alkaline by the seventh day of incubation, and the deanimation was quite far advanced. At first sight this might seem to be at variance with the usual effect of utilizable carbohydrate upon the nitrogenous metabolism: the cause of this apparent discrepancy, however, is the exhaustion of the carbohydrate quite early in the growth of the microbes.[25] In milk, where the percentage of carbohydrate is much greater this sparing action is clearly shown even after seven days' growth.

SUMMARY AND CONCLUSIONS

The results of the chemical and bacteriological study of the 50 specimens of duodenal contents obtained from a consecutive series of adults, recorded herewith, afford some substantiation for each of the prevailing theories of the microbic content of this part of the alimentary tract. Approximately one half of the specimens obtained failed to yield significant numbers of microorganisms in artificial cultural mediums; the remainder varied from very moderate cultivations, yielding bacteria that were chemically nearly inert except for acid production, to heavy growths, reminiscent of those that would have confidently been anticipated, had the specimens come from the lower levels of the small intestine.

No direct evidence of a microbicidal action of the duodenum or its contents was detected, though many specimens failed to show bacteria,

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[25] Kendall, Day and Walker: J. Am. Chem. Soc., 1913, 35, p. 1234.

even upon direct microscopic examination. This does not however, in itself preclude this possibility, although the positive evidence deduced from the readiness with which many duodenal specimens yielded viable organisms is rather against it.

The relative abundance of members of the mocosus-capsulatus group, of the enterococcus and of staphylococci in the specimens studied suggests these organisms are rather easily adaptive to the adult duodenal environment.

The occurrence of gas bacilli in three of the duodenal specimens indicates that this organism is to be regarded as a potential resident of the duodenum. The number of cases is far too small to permit of generalization, but it is surmised that one essential condition for an exuberant growth of gas bacilli is a low acid content in the duodenum. In the cases cited the low acidity was due both to a paucity of cultivable lactic acid producing bacteria and to an overgrowth of alkali producing microbes. Further study of such cases is much needed.

THE RELAXATION OF HISTAMINE CONTRACTIONS IN SMOOTH MUSCLE BY CERTAIN ALDEHYDES

STUDIES IN BACTERIAL METABOLISM. LXXXIII.

Arthur Isaac Kendall

From the Department of Bacteriology and Public Health, Washington University School of Medicine, St. Louis, Mo.

An extended series of experiments [1] has shown that a small amount of a neutral formalin solution will cause an almost complete relaxation in a piece of guinea-pig intestine, or uterus, that has been caused to contract by histamine, or the histamine-like substance produced by many strains of the gas bacillus.[2] This relaxation takes place as abruptly, but of course in the opposite direction, as the contraction itself.

The relaxation of contractions produced in smooth muscle by histamine and by histamine-like substances is by no means limited to aldehydes. It has long been known, for example, that adrenalin will relax histamine contractions very promptly, and pharmacologists are familar with other substances that have a similar effect. It is quite significant, however, that formaldehyde interferes with the adrenalin action, as Cramer [3] predicted some years ago.

Formaldehyde in small concentration ($1 : 750$ or less), unlike histamine, is without obvious effect upon smooth muscle, hence it seems reasonable to attribute this striking phenomenon to a chemical change in the histamine molecule itself and of such a character as to destroy its contracture-inducing power for smooth muscle. Further evidence in support of this view is afforded by the fact that the aldehyde-histamine complex can be washed out from smooth muscle in which contraction has taken place and relaxation has been brought about, and the process repeated twice or even thrice without much impairment of the initial height of contraction.

It will be recalled that Sörenson [4] used the formol titration to estimate the amount of amino nitrogen in various protein derivatives, and it is not improbable that the same reaction takes place between histamine and formaldehyde as would occur between histidine and formaldehyde, in accordance with the following equation:

$$R.CH_2. CH_2NH_2 + H.CHO = R.CH_2. CH_2N : CH_2 + H_2O.$$

[1] Kendall: Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 316.
[2] Kendall and Schmitt: Ibid., 1926, 24, p. 104.
[3] J. Physiol., 1911, 42, p. 5.
[4] Biochem. Ztschr., 1908, 7, p. 407.

If this is the case, then other aldehydes also might be expected to relax contractions induced in smooth muscle by histamine, and histamine-like substances, provided, of course, the aldehyde molecule can pass through the intervening structures to the muscle itself.

A number of aldehydes and related compounds have been tried, using the Trendelenberg technic [5] which has been found quite satisfactory for experiments of this type,[6] to test this hypothesis. The list studied comprised the following: formaldehyde and its polymer, trioxymethylene; acetaldehyde and its polymer, paraldehyde;[7] glycol and benzaldehyde, and glyceric aldehyde, methyl glyoxal and polyglyoxal; chloralhydrate, and aldol, all of the aldehyde series; and acetone, dihydroxyacetone, acetophenone, and a series of tri, tetra, penta and hexose aldose and ketose sugars.

For simplicity of discussion, it may be stated at once that the carbohydrate aldoses and ketoses, as perhaps might be surmised, had no appreciable effect in reducing the histamine contraction: acetone, dihydroxyacetone, trioxymethylene and choral hydrate were also ineffective.

On the other hand, it was somewhat surprising to discover that methylglyoxal and polyglyoxal, both of which were tried up to twice molar concentration, were inert. Formaldehyde, acetaldehyde, paraldehyde, aldol, and even acetophenone were regularly effective in relaxing the histamine contracture. The speed of the reaction seemed to be somewhat slower with increasing size of the molecule. Paraldehyde was the slowest of all, presumably because of the time required for depolymerization to acetaldehyde. This statement, however, is made upon the assumed correctness of the current belief that paraldehyde is a cyclic compound in equilibrium with acetaldehyde, of the type

$$
R.CH
\begin{array}{c}
O - CH\,(R) \\
\diagup \qquad\qquad \diagdown \\
\qquad\qquad\qquad O \\
\diagdown \qquad\qquad \diagup \\
O - CH\,(R)
\end{array}
$$
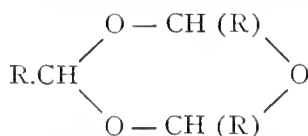
It is not without significance that this substance bears a structural resemblance to the piperazines as for example, 3.6 dimethyl 2.5 dioxo-

$$
\text{piperazine}\quad HN
\begin{array}{c}
CH.CH_3.CO \\
\diagup \qquad\qquad \diagdown \\
\qquad\qquad\qquad NH. \\
\diagdown \qquad\qquad \diagup \\
CO.CH.CH_3
\end{array}
$$ These have considerable theoretical
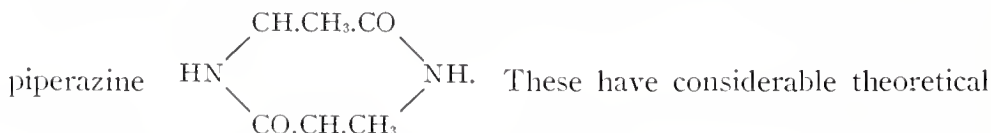
[5] Arch. f. exper. Path. u. Pharmacol., 1917, 81, p. 53.
[6] Kendall and Schmitt: J. Infect. Dis., 1926, 39, p. 250.
[7] Meyer and Jacobson: Lehrb. d. Org. Chem. 2 Aufl. 1923, 1, p. 676.

interest in that they may exist as ring compounds of certain amino acids in the protein molecule.[8] It is by no means impossible that substances of this type, containing aromatic nuclei, and even reacting like aromatic amines, may be freed from proteins under the influence of slight enzymic cleavage.[9]

One of the outstanding general features of this series of experiments was the very prompt response on the part of the muscle to the reactive substance, be it either histamine, or histamine-like substance of the gas bacillus causing the contraction, or the aldehyde in relaxing the contraction. This response, indeed, was well near instantaneous as is quite clearly shown in the kymograph records below. Nevertheless the amounts of reactive substance in each instance were very small. The greatest actual concentration used was 0.2 cc. aldehyde in the bath which held 150 cc. The amount of histamine was less than one part in hundreds of thousands. It would appear that the reactive substance is drawn very rapidly to the smooth muscle. Whether this is due to specific adsorption cannot be told from these observations.

Methods: The Trendelenberg[5] technic was used throughout these experiments. The Tyrode solution was alkaline in reaction (P_H 7.9—8.0) and, because of its buffer content, practically unaltered in reaction by the small amounts of neutral reagents added. The bath held 150 cc. and was kept at 37—38 C. by a thermostat. Oxygen was bubbled through continuously.

The upper two thirds of the guinea-pig intestine was used, or the uterus. The organ was removed from the animal as soon as possible, and placed in a large Petri dish, both halves of which were covered with filter paper and moistened with Tyrode solution. This was kept in a cold room at 40 C. until actually used.

The guinea-pigs were not fed the day of the experiment, experience having shown that the empty gut was cleaner and more constant in its reactions than the full gut. The animals were killed by a blow on the skull.

Records of contractions and relaxations were made in the usual manner on a kymograph, with a time marked indicating three second intervals. The ratio of lever arms was 8 : 1.

Experiments with aldehydes were performed in each of two ways: first the appropriate amount of the aldehyde, or similar solution, was introduced into the Tyrode bath containing the muscle, and then after a few seconds to see if the muscle remained quiescent, the requisite amount of histamine, or gas bacillus filtrate was added. The second method was to add the contracture-inducing substance first, and then at the proper time, the contraction-relaxing substance. This could be and frequently was, done at a time when the muscle was still shortening, to be quite certain that the relaxation was due to the action of the aldehyde rather than failure of the reagent inducing contraction.

Exper. 1.—The first series was invariably negative, when concentrations of aldehyde not less than 1 : 750 were present in the bath. This is to say, neither

[8] Fischer: Ber. d. deutsch. Chem. Gesellsch., 1905, 38, p. 607; Fischer and Abderhalden: Ibid., 1906, 39, p. 2315.

[9] Kendall and Keith: J. Infect. Diseases, 1926, 38, p. 193.

the aldehyde, nor the histamine nor the gas bacillus filtrate caused any change in the length of the muscle. Of course if the substance added did not prevent the action of the histamine, for example acetone, contraction would occur. None of the aldehydes that relaxed contractions, however, failed to prevent the histamine contraction from taking place. The records of the first series are omitted, because of course they show no muscular change. It is worthy of note that those aldehydes that did prevent histamine contractions could be washed out quantitatively from the muscle and from the bath with fresh Tyrode solution. Then histamine caused the usual contraction to take place. Obviously, the muscle is not seriously injured by contact with the aldehyde under the conditions of the experiment.

Exper. 2.—The second series of experiments, from which characteristic kymograph records are herewith presented, was performed in the reverse order. First the piece of intestine, or uterus, was caused to contract either with filtrate from the gas bacillus, or histamine solution, then aldehyde, or other substance was added and the effect recorded. For this series of experiments a uniform amount of the substance 0.2 cc. (neutralized, if necessary), was added to the Tyrode solution, making therein a final concentration of 1 : 750. A subsequent report will deal with the minimal quantities sufficient to reduce histamine contractures. It can be stated now that these amounts will be less than one third those used here.

Exper. 3.—A third experiment was tried: a contraction-inducing amount of histamine was mixed with a small but excessive amount of formalin. The mixture was without obvious effect upon the gut; but multiple amounts of histamine were not completely neutralized by formalin. The several factors involved in this phenomenon are beyond the scope of this communication; they will be discussed later.

It must be borne in mind that the reactions considered here take place in a buffered solution having a slightly alkaline reaction: (P_H 7.9—8.0) aldehyde-ammonia reactions [10] and the formal titration [4] take place at this point.

Judging from the literature commonly quoted, but little attention has been paid to aldehyde-protein and aldehyde-protein derivative reactions. This is not surprising when it is remembered that the substances studied in this connection have not been associated with potential pharmacological activity.

Bischoff and Reinfeld [11] did however, prepare compounds from the interaction of putrescin and cadaverin with formaldehyde, and Rügheimer [12] prepared compounds resulting from the interreaction of primary amines with higher aldehydes. Fischer and Warburg [13] actually utilized formic acid in the synthesis of certain peptides. Schwartz [14] made the interesting observation that formaldehyde, and to a lesser extent, acetaldehyde reacted with serum albumin and serum globulin to form definite compounds, which have some forty aldehyde groups for each one hundred nitrogen atoms of the molecule. These new substances gave the biuret, Millon and xanthoproteic reactions, but not the Molisch, Adamkiewz or Liebermann test. They were digested by pepsin but not by trypsin. It has long been known, also, that formaldehyde in dilute solution slowly unites with proteins. The color reactions elicited, supposed to be compounds of the aldehyde with

[10] Richter-Smith: Organic Chemistry, 1900, 1, p. 205.
[11] Ber. d. deutsch. chem. Gesellsch., 1903, 26, pp. 35, 41.
[12] Ibid., 1906, 39, p. 1653.
[13] Ibid., 1905, 38, p. 3997.
[14] Ztschr. f. physiol. Chem., 1900-1901, 31, p. 460.

Figs. 1, 2 and 3.—Three successive contractions and relaxations induced in the same piece of guinea-pig intestine. Intestine washed with fresh Tyrode solution after each relaxation. Figure 1: contracture induced by 0.2 cc. filtrate of gas bacillus; relaxed with 0.1 cc. paraldehyde. Figure 2: contracture induced by 0.1 cc. histamine solution; relaxed with 0.1 cc. formaldehyde. Figure 3: contraction induced by 0.1 cc. histamine solution; relaxed with 0.1 cc. paraldehyde.
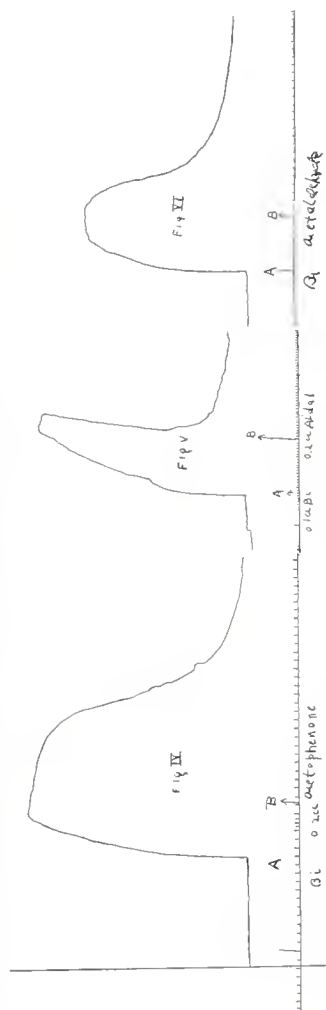
Fig. 4.—Intestinal contraction induced by 0.1 cc. histamine solution; relaxed with 0.2 cc. acetophenone.

Fig. 5.—Intestinal contraction induced by 0.1 cc. histamine solution; relaxed with 0.2 cc. aldol.

Fig. 6.—Intestinal contraction induced by 0.1 cc. histamine solution; relaxed with 0.1 cc. acetaldehyde.

(All figures are reduced to one-fourth original size.)

tryptophane, have been studied by Komm.[15] Meyer and Jacobson[16] state that primary amines react with formaldehyde in alkaline solution, and further that methyl imidazole and formaldehyde, kept for eight hours at 120 C. unite to form 4.5 methyl methylol imidazole.[17] Langheld[18] has studied somewhat similar interreactions of imidazole and acetaldehyde. It is worthy of note that in none of these citations does it appear that pharmacologically active substances, as for example the aromatic amines, were considered, although the nature of the compounds suggests that such reactions might occur. Hence probably the acceptance of the facts, but with no particular interest in the possibilities of the reactions and their significance.

The principal inference to be drawn from these several comments is not one of censure, but rather of the remarkable interdependence of apparently distantly related sciences. Organic chemistry, physiology and pharmacology are brought into juxtaposition in this instance through bacteriology.

DISCUSSION

The subjoined reproductions of the kymograph records show the essential features of these experiments. The speed with which the muscle contracts in response to the stimulation of the histamine or gas bacillus filtrate, and the speed with which the contracted muscle relaxes, is shown by the upward, and the downward course of the tracings, respectively. The time intervals are uniformly three seconds. The outstanding feature is the truly amazing rapidity with which the reactive substance passes into solution, traverses the serous coat and either stimulates the muscle to contract, or to relax, as the nature of the reagent directs. The law of multiple proportions does not hold, however, as was stated previously. A mixture of histamine and formalin, containing merely enough of the amine to cause, by itself, a maximal contracture, and a small excess of formalin, will usually produce none, or at most, a very slight muscular contraction in the gut. Ten times the amounts of the reactive substances will show almost invariably a moderate contraction, indicating presumably some unneutralized histamine. This suggests that the reaction between the amine and the aldehyde is of the nature of a weak base and a weak acid. It must be admitted that this explanation is not wholly satisfactory, however, because relatively more aldehyde is required to reduce this "residual contraction" than would ordinarily suffice to relax a much smaller amount of free histamine. Nevertheless, the relaxation of histamine contractures by certain aldehydes seems unequivocal, under the conditions of these experiments.

[15] Ibid., 1926, 156, p. 35.
[16] Lehrb. d. org. Chem., 2, Aufl. 1913, 1, p. 753.
[17] Ibid., 2, p. 446.
[18] Ber. d. deutsch. chem. Gesellsch., 1909, 42, p. 2373.

It should be stated in passing that other substances, as certain kinds of peptones, the depressor-like substance found in certain urines, certain commercial tissue extracts and hormones, and certain slightly cleaved proteins may contain products which cause histamine-like contracture in guinea-pig gut. These contractions are almost always relaxed by certain aldehydes, although not always completely. The contraction caused by the reinjection of the homologous protein to a strip of intestine from a sensitized guinea-pig is also relaxed, or prevented from contracting, as the aldehyde is added before or after the protein. This will be discussed in detail later.

Turning now to the aldehydes and related substances that seem to be potent in relaxing histamine contractions, it will be seen from the table below that there is a very definite relationship between the relaxing property and the structure of the entire aldehyde molecule. Briefly, the relationship may be expressed thus, in so far as the small series herein reported is concerned. Oxygen attached to the carbon atom next the aldehyde group causes the aldehyde group to be inactive; fully hydrogenated carbon next the aldehyde group in so far as these experiments show, is compatible with aldehyde activity.

Inactive aldehydes.

$$
\begin{array}{lll}
CH_2OH & CH_2OH & CH_3 \\
| & | & | \\
| & | & | \\
CHO & CHOH & C=O \\
\text{Glycol} & | & | \\
\quad\text{aldehyde.} & | & | \\
& CHO & CHO \\
& \text{Glyceric} & \text{Methyl} \\
& \quad\text{aldehyde.} & \quad\text{glyoxal.}
\end{array}
$$
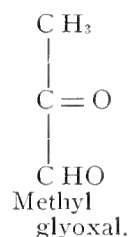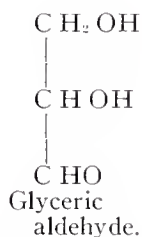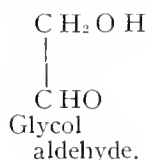
There is oxygen attached to the carbon atom next the aldehyde group. Dihydroxyacetone, acetone and chloral hydrate were also inactive Indeed, ketones, except acetophenone, were without effect.

Active aldehydes.

$$
\begin{array}{lll}
H.CHO & CH_3 & CH_3 \\
& | & | \\
& | & | \\
\text{Formaldehyde.} & CHO & CHOH \\
& & | \\
& \text{Acetaldehyde.} & CH_2 \\
& & | \\
& & | \\
& & CHO \\
& & \text{Aldol.}
\end{array}
$$
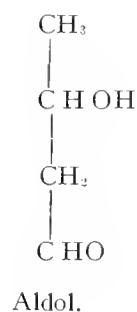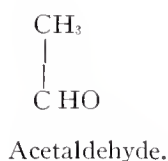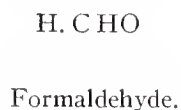
There is no oxygen attached to the carbon atom (except formalde-
hyde) next the aldehyde group. Paraldehyde and acetophenone were
also reactive in reducing the histamine contractions.

It will be seen that aldol is a very important member of this series.
The carbon atom twice removed from the aldehyde group which has an
oxygen atom attached, has no discernible influence upon the activity of
the compound, whereas glycol aldehyde, in which the oxygen containing
carbon is adjacent to the aldehyde group, is quite inert. Benzaldehyde,
however, was apparently unable to reduce the histamine contracture.
This substance stands in direct contrast to acetophenone. The few com-
pounds studied, therefore, point to the interesting hypothesis that reduced
carbon next the aldehyde group favors the neutralization of the
contraction-inducing power of physiologically active ethyl amine deriva-
tives, at least in aldehydes of relatively small molecular weights, and
that oxidized carbon next the aldehyde group makes the aldehyde group
inactive toward these ethyl amine derivatives. Fourneaux [19] cites a
somewhat similar relationship between physical activity and oxidized
carbon in the group of ortho, meta, and para hydroxethyl amines.
When the hydroxyl group is in the meta or para position with respect to
the amino group, physiologic activity is relatively great. When the OH
group is adjacent, that is, in the ortho position, the physiologic activity is
much less.

On the other hand, with respect to amines, neither methyl nor ethyl
amine, even in considerable concentrations, has more than minimal
contraction inducing power for smooth muscle. Attached to a phenyl
group, the activity at least for guinea-pig gut is not very much increased;
attached in the beta position to the imidazole nucleus, the reactivity is
magnificent. One might expect that allantoin, which is an imidazole urea
derivative, should be physiologically active, and in virtue of its amino
group induce contractions in surviving guinea-pig intestine, but it appears
to be wholly inert. It seems not improbable that the proximity of the
oxygen to the amino group may be a determining factor, much as oxygen
similarly placed reduces the activity of aldehyde groups, and of ortho
phenylethyl amine. In this connection, it is worthy of mention that
neither acetone nor dihydroxy acetone is able to reduce histamine con-
tracture, but, on the other hand, acetophenone is nearly as potent as
formaldehyde.

It is quite clear that this entire question of amines and aldehydes,
both highly important chemical groups in the animal and human body,

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[19] Organic Medicaments and Their Preparation, 1925, p. 125.

may be studied from a new angle through the induction and reduction of smooth muscle contracture. It follows because of these rather definite relationships manifested between closely related compounds, in respect to their pharmacological actions, that a detailed study of a complete series of these substances should throw light upon the question of relations between structure and activity.

SUMMARY

The relaxation, by certain aldehydes, of contractions induced in guinea-pig gut and uterus by histamine and certain histamin-like substances, has been demonstrated graphically by kymograph tracings. This reaction is not due exclusively to the aldehyde group, per se, but is markedly influenced by the composition and structure of the aldehyde molecule as a whole.

The absence of oxygen on the carbon atom next the aldehyde group appears to predispose the aldehyde molecule as a whole toward reactivity. The presence of oxygen on the carbon atom next the aldehyde group restrains, or prevents, the reactivity of the aldehyde radical.

It must be emphasized that the evidence of aldehyde activity presented herewith is based solely upon physiologic response induced in smooth muscle. It does not necessarily follow that these reactions would take place quantitatively in the test tube, although chemical literature contains many instances where such aldehyde amine reactions have been investigated.

The method lends itself to the detection of free histamine and histamine-like substances. Many of these products found in peptones, proteoses, tissue extracts, blood, urine and hormones, exert a depressor action upon blood pressure, but fail to induce contracture in the guinea-pig gut.[20]

Finally, it must be distinctly recognized that substances other than amines cause contracture in guinea-pig gut, and it is equally certain that substances other than aldehydes, for example, adrenalin, relax these contractures.

[20] Guggenheim and Löffler: Biochem. Ztschr., 1915-1916, 72, p. 303, have used guinea-pig gut to detect the presence of amines.

TYPES OF HEMOLYTIC STREPTOCOCCI IN CERTIFIED MILK

William D. Frost, Mildred Gumm

AND

Robert C. Thomas

From the Agricultural Bacteriological Laboratories of the University of Wisconsin, Madison

In a paper by Brown, Frost and Shaw [1] it was shown that the available cultures from various epidemics of septic sore throat, eleven in all, were identical as determined by certain cultural tests. For this species, or variety, the name Streptococcus epidemicus Davis was used. It was also shown that the hemolytic streptococci ordinarily found in certified milk were quite distinct from this organism. These milk streptococci were regarded as of bovine origin, harmless for man and easily distinguishable by cultural characteristics from Streptococcus epidemicus. A routine method was suggested for the examination of milk, with the idea that by its use cows infected with Streptococcus epidemicus, if such should exist, could be recognized and removed from the herd before they could become a menace to the milk consumers.

The present paper deals with this routine method somewhat more in detail and gives the results obtained by its use on 1,304 group samples of milk and extending over the period of a year and a half.

Routine Procedure for Detection and Differentiation of Types.—Collection of Samples: The samples are obtained usually between milking times. The udders are washed and the first two streams from each quarter are discarded. Then a few streams from each quarter are collected in a two ounce bottle. These bottles have cork stoppers and are sterilized in the hot air oven. The milk from ten cows is put in each bottle. After collection the samples are immediately placed on ice in the carrying case. About 25 samples are taken on one trip and are taken to the laboratory as soon as possible. In the work here reported they were plated usually within four hours after collection.

First Day—Plating: One cc. of the milk is mixed with 19 cc. of sterile tap water, and to 1 cc. of this 1:20 dilution, 10 cc. of veal agar, containing 0.7 cc. of horse blood is added, and is spread in 10 cm. Petri dishes. The plates are incubated for about 24 hours at 37 C.

Second Day—Examination: The plates are examined in 24 hours and if hemolytic colonies are present they are counted. Sometimes the plates are too thickly seeded to be of value, in which cases higher dilutions are made of the milk which in the meantime is kept in the refrigerator. Representatives of

[1] J. Infect. Dis., 1926, 38, p. 381.

all the beta colonies are picked into tubes of veal broth to which about two drops of sterile serum has been added. Sometimes there are two or more different types of colonies present on the same plate. Plates not showing hemolytic colonies or on which the colonies are not well developed are incubated another day, and then treated as above. At this time the total count is also recorded. The colonies of Streptococcus epidemicus are quite characteristic. The hemolytic area is of considerable size and clear. The deep colonies are rather large and the surface colonies are especially so. Moreover they have a moist appearance which is characteristic. Experience enables one to recognize these with a considerable degree of confidence. Such colonies should always be looked for and if such are found microscopical preparations should be prepared with moist India ink and capsules searched for. If capsulated streptococci are found at this point the cows whose milk made up the composite sample in which they were found should be examined individually without delay.

Third Day—Hemolytic Titer: One-half cc. of the broth cultures made the day before are mixed with the same quantity of a 5% suspension of rabbit blood corpuscles in a three inch test tube and incubated at 37 C. for two hours. Those tubes which show little or no hemolysis are discarded at this point. From the original broth tubes of those cultures which hemolyze in the test tube inoculations are made into sodium hippurate broth, dextrose broth and onto blood agar slants.

Fourth Day—Examination for Capsules: Capsules are most likely to be found in young blood agar slants, which, it is important to note, must have been incubated in a moist atmosphere. Moist India ink preparations are the most satisfactory. The presence of a capsule puts any streptococcus under suspicion.

Fifth Day: Determination of the hydrogen ion concentration of the dextrose serum broth may be made at this time but never in less than 24 hours. If the P_H is as low as 4.5 the cultures may be discarded. The ferric chloride test for the hydrolysis of sodium hippurate may be made at this point and if positive the culture may be discarded. Any culture not discarded up to this point should be inoculated into serum fermented veal broths containing respectively the following carbohydrates: lactose, saccharose, mannitol and salicin. Incubate at 37 C. for five days.

Tenth Day: One or two drops of methyl red is added to the five day old serum broth cultures containing the test substances. If the color is red the reaction is positive; i. e., fermentation has occurred. If the color is yellow, one or two drops of phenol red are added, in which case if the color is red the fermentation is negative. If the color is not red with either indicator, the P_H is determined definitely. When the P_H is less than 6.5, it is regarded as positive.*

Should any of these cultures prove to be Streptococcus epidemicus, the cows whose milk made up the composite sample in which this organism was found should be examined individually without delay, the infected cow found and removed from the milking line.

Quantitative Results.—The proportion of samples showing beta hemolytic streptococci is shown in table 1. It will be noticed that

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* The LaMotte hydrogen ion testing set, devised by Brown, has been used in all our work.

1,304 group samples have been analyzed and that 903 of them, or 69.2%, showed beta hemolytic streptococci. In 122 group samples more than one type of beta colony appeared. Of the 1,065 beta colonies picked, only 394, or 37%, hemolyzed in the test tube. These we refer to as true betas. They are the only ones we are interested in in this paper.

Attention should be called to the fact that this does not mean that as large a proportion of the individual cows carry beta streptococci as would be indicated by these figures. What it does mean is that in 69%,

TABLE 1

Beta Colonies from Group Samples of Certified Milks

	Number	%
Group samples analyzed......................................	1,304	100
Samples showing beta colonies (several samples had more than one species present)	903	69.2
Samples showing true beta colonies..........................	373	28.6
Total number of beta colonies picked........................	1,065	100
True betas (hemolyzing in test-tube)..........................	394	37

TABLE 2

Quantitative Distribution of Streptococci, of the True Beta Type, in Group Samples of Certified Milk

	Number	%
Samples showing true beta colonies............................	373	100
Samples showing, per cc.:		
1 to 20..	38	10.2
21 to 60..	24	6.4
61 to 100...	18	4.9
101 to 500..	112*	30
501 to 1,000..	52*	13.9
1,001 to 10,000...	105*	28.1
10,001 to 100,000...	21	5.6
100,001 to 1,000,000..	2	0.5
Over 1,000,001		

* Streptococcus epidemicus present.

of any group of ten cows, at least one of these ten cows was shedding hemolytic streptococci. The only way to get at the percentage of individual cows carrying these germs is to examine the milk from all the cows individually rather than in groups. In some previous work on these same herds, where the individual cows were tested, Frost and Bachmann [2] have shown that only about 10% of the cows shed hemolytic streptococci of the beta type.

[2] Am. J. Pub. Health, 1923, 13, p. 300.

The relative abundance of true beta streptococci in the group samples is shown in table 2. A study of this table shows that in slightly over 10% of the samples, the number of hemolytic streptococci present in the milk was 20 per cc. This means that there was only one colony on

TABLE 3

Types of Beta Streptococci Found in Certified Milk and Their Relation to Recognized Species

Num-ber	Action on				PH in Dex-trose	Sodium Hip-pu-rate	Cap-sule	Name	Group (B. F. & S.)*	Cultures from Milk
	Lac-tose	Su-crose	Man-nitol	Sali-cin						
1	+	+	+	+	4.9	0	0	Streptococcus infrequens		
1a	+	+	+	+	4.5-5.0	0 slight	0	Streptococcus infrequens	3	114
2	+	+	+	0	5.1-5.9	+	0	Streptococcus hemolyticus i		
2a	+	+	+	0	5.1-5.9	+ slight	0	Streptococcus hemolyticus i	..	3
3	+	+	0	+	5.1-5.9	0	+	Streptococcus epidemicus		
3a	+	+	0	+	4.9-5.2	0	+	Streptococcus epidemicus	1	4
4	+	+	0	+	5.1-5.4	0	0	Streptococcus pyogenes Streptococcus pyogenes		
5a	+	+	0	+	4.4-4.9	+	0	Streptococcus mastitidis	2	186
6	+	+	0	0	5.1	0 slight	0	Streptococcus anginosus		
6a	+	+	0	0	4.6-5.1	+	0	Streptococcus asalignus n.sp.	4	49
6b	+	0 slight	0	0	4.7-5.5	+	0	Streptococcus ?	5 and 6	15
7	0	+	+	+	..	..	0	Streptococcus alactosus (hemolyticus ii)		
8	0	..	+	0	..	..	0	Streptococcus hemolyticus iii		
9	0	..	0	+	..	..	0	Streptococcus equi		
10	0	..	0	0	..	..	0	Streptococcus subacidus		
10a	0	0	0	0	4.7-5.2	+	0	Streptococcus subacidus		8

Miscellaneous cultures not falling in above classification.................................... 4
Cultures lost before typing was complete.. 11

394

* Brown, Frost and Shaw.[1]

the plates, for the dilution of milk in all these cases was 1 to 20. If a greater dilution had been used, a considerable number of these would have been missed.

It will also be seen that over 90% of the samples had less than 10,000 of these true beta streptococci per cc.

Types or Species of Hemolytic Streptococci Found in Certified Milk.—The cultures which we have isolated are grouped according to their action on the various test substances in table 3. The first lines of the groups, numbered serially without letters, indicate the recognized species (Holman [3] and Brown [4]). No attempt is made here to settle the question whether or not these are really species or merely varieties. We do find, however, that with our technic the differences which are used to separate these types have been constant and we prefer for the time being to refer to them as species rather than varieties. The other lines in table 3 numbered with letters represent the cultures obtained by us from milk.

We have failed to find among our cultures representatives of all of the recognized types. It is interesting, however, to note that those which are missing are largely those which do not ferment lactose, while all of the recognized types that are known to ferment lactose have been found. This suggests that these organisms are present because they grow readily in the milk. They may maintain themselves while in the udder on the milk and not on the animal tissue.

It will be noticed also that the streptococci we have found in milk fall into 6 species as follows:

Streptococcus mastitidis .... 186 cultures
Streptococcus infrequens .... 114 cultures
Streptococcus asalignus N. Sp...................... 64 cultures
Streptococcus subacidus .... 8 cultures
Streptococcus hemolyticus i....................... 3 cultures
Streptococcus epidemicus .... 4 cultures

Streptococcus Mastitidis: Streptococcus mastitidis has been most frequently found in our work. Practically one-half of all our cultures belong here. In most of its characters it resembles Streptococcus pyogenes but differs very definitely in that it is a high acid producer in dextrose (P_H around 4.5) and also very definitely hydrolyzes sodiums hippurate which Streptococcus pyogenes never does.

This streptococcus may be etiologically associated with mammitis in cows but in our experience there is little if any relation between the presence of this germ in the milk and clinical mammitis and it seems very certain that there is no reason for regarding this organism as in any way harmful for man.

Streptococcus Infrequens: The second most common group into which our cultures fall contains Streptococcus infrequens. Of our 394

[3] J. M. Research, 1916, 34, p. 377.
[4] Monograph 9, Rockefeller Inst. for M. Research, 1919.

cultures, 114 or nearly 28% are in this group. These cultures are all very similar and differ from Streptococcus infrequens, reported from human sources, only in producing slightly more acid in dextrose and in having a slight tendency to hydrolize sodium hippurate. But these tendencies are not as marked as they are in the preceding group which led to the separation of Streptococcus mastitidis from Streptococcus pyogenes. It is probably impossible, in the present state of our knowledge to dogmatize in regard to these relationships. Attention may be called to the fact, nevertheless, that if the two members of this group are the same organism then it belies its name for it is not infrequent but rather the second most frequent of our milk cultures. It seems quite possible that the reason why it was found so infrequently in human infections is because it is in reality a bovine species and is present in man not as an etiologic factor in disease but merely by accident, perhaps from drinking milk. If on the other hand, it is found that the differences are constant and especially if it is found that there are other and more important differences between the two strains, then our milk strain needs a new name. This possibility was suggested in our earlier paper.[1]

Streptococcus Anginosus and Asalignus: The third most numerous group contains Streptococcus anginosus. This species is well differentiated from those already considered since it is not able to ferment either salicin or mannitol. Its name indicates that it is a throat organism. Our milk cultures differ from this human type in that they are high acid producers and hydrolyze sodium hippurate. We have regarded these two characteristics as sufficient to differentiate Streptococcus mastitidis from Streptococcus pyogenes and for the same reason, our cultures here are regarded as different from Streptococcus anginosus. So far as we are aware, this is the first time that the identity of this organism has been recognized and we suggest the name Streptococcus asalignus. As will be noted, about one-fourth of these differ from the other three-fourths in the effect on saccharose. It seems doubtful whether this is a sufficient difference to rank as specific and we have included them with the others.

The next two groups are represented by very few cultures: Streptococcus subacidus claims 8 cultures and Streptococcus hemolyticus i 3 cultures.

Streptococcus Epidemicus: We have found this organism in four cows during this last year. In our regular monthly examinations, we found in the March group samples from one of the farms a nencapsulated streptococcus on the third day after the samples were col-

lected. We immediately went to the farm and took individual samples from the ten cows whose milk made up the group sample. At the same time we requested that the milk from this group of cows be kept out of the city supply until the particular cow or cows could be located. The laboratory tests showed that only one cow of this group was shedding hemolytic streptococci. But these proved to be Streptococcus epidemicus. The other cows of this group were put back in the herd and the infected cow permanently removed and ultimately brought to the University isolation barns for study. This cow had recently freshened but at no time had garget been observed in the udder. A physical examination showed a slight tenderness in one quarter and two small abscesses between the bases of the teats. These sores were nearly healed at the time but a small amount of pus was expressed and cultured. There were two kinds of streptococci present and no other organisms. Both of these cultures, however, failed to hemolyze in the test tube and their study was not carried further. The infection in this cow was confined to one quarter. When first discovered this cow was shedding relatively large numbers of this organism, nearly two million of these bacteria per cc. of the mixed milk from all quarters and once several months later the number was about 350,000 per cc. but for the most part in the examinations that were made each fortnight over a period of six months these hemolytic streptococci averaged less than 30,000 per cc.

In July one of our routine samples from a bottle ready for market showed the presence of an encapsulated organism. We then went through the herd, found this organism in one of the group samples and then through the individuals in this group and located the cow. This cow was immediately sent to the packing house. When we had her located she was giving off these organisms at the rate of 700 per cc.

In October we found the third cow, which had freshened recently, had just been purchased and put in the milking line. We found three infected quarters in which there were a moderate number of Streptococcus epidemicus. She was brought to the University of Wisconsin isolation barns and has been under observation since. There has been little change in the condition of the udder during these four months.

In November we detected the fourth cow, which had recently been purchased from a dealer and had freshened on the farm since her arrival. This cow which had just gone into the milking line was immediately isolated but within a few days the udder stopped shedding Streptococcus epidemicus. The cow was disposed of at this point.

Summarizing: Two of these four cows carried this infection for several months at least. The first one for about 6 months, the second one for at least 4 months. No attempt was made to study the third, but the infection in the fourth cleared up almost immediately. Because of the persistence of the infection in two of the cows we advise the immediate and permanent removal of any and all cows showing infection with Streptococcus epidemicus from milk producing herds.

SUMMARY

The milk from about 1,000 cows on 5 different certified farms has been plated on blood agar about once a month throughout the period of 1½ years.

The samples were from groups of 10 cows. Hemolytic streptococci of the true beta type appeared in 28.6% of the samples. The number of such streptococci in the samples were usually very small, less than 1,000 per cc. in 65%, and less than 10,000 per cc. in 94% of the samples.

Classification of 394 cultures of the true beta type, by means of 7 different tests, placed them in 6 different species. Five of these, the mastitidis, infrequens, subacidus, hemolyticus i and epidemicus have been described before. One new species is described, Streptococcus asalignus.

All of the streptococci found in certified milk, and identified, are of the bovine type with exception of 4 cultures which proved to be Streptococcus epidemicus Davis.

The method of procedure used in this work for examining milk and identifying streptococci promises to be of distinct value in the production of milk of the highest quality.

THE PRECIPITIN REACTION OF FIBRINOGEN

LUDVIG HEKTOEN AND WILLIAM H. WELKER

From the John McCormick Institute for Infectious Diseases and the Laboratory of Physiological Chemistry, University of Illinois, College of Medicine, Chicago

Comparatively little attention has been paid to fibrinogen in immunologic work. On account of its antibodies and being itself antigenic, the immunologist has interested himself especially in the serum and its proteins. Our ideas about antigenic animal specificness have developed mainly on the basis of the species limitations of the immune reactions of serum and its constituents. The question whether fibrinogen follows the same rule as other blood proteins has not been raised until recently.

Nolf [1] observed that the serum of rabbits injected with chicken plasma caused instant precipitation when mixed with chicken serum. To study fibrinolysis, Camus [2] injected rabbits with powdered dog fibrin. The serum of the rabbits did not dissolve fibrin, but caused precipitation in the fibrin suspension, in dog serum, and in solution of dog fibrin ferment. And the serum of rabbits injected with dog serum precipitated dog serum and fibrin suspension. Bordet and Gengou,[3] in their study of anticoagulant serums, found that the serum of guinea-pigs immunized with rabbit plasma not only neutralized fibrin ferment in rabbit serum, but also caused flocculation without coagulation in rabbit plasma. This antiserum had a certain effect on fibrinogen, but like the antifibrinogen serum of Camus, it was not fibrinolytic. As the antiserum neutralized the fibrin ferment in other serums than rabbit, it was suggested that fibrin ferment is not species-specific. Gengou [4] next showed that, in rabbits, horse fibrinogen prepared by Hammarsten's method changed the serum so that it produced an immediate and copious precipitate in the fibrinogen solution.

Interest in the antigenic properties of fibrinogen now became latent. In 1912 Bauer and Engel [5] report that beef and swine fibrinogens, prepared by Heubener's modification of Hammarsten's method, by comple-

[1] Nolf, P.: Contribution a l'Étude des Sérums Antihématiques, Ann. de l'Inst. Pasteur, 1900, 14, p. 296.

[2] Camus, L.: Recherches sur la Fibrinolyse, Compt. rend. Acad. de sc., 1901, 132, p. 215.

[3] Bordet, J., et O. Gengou: Recherches sur la Coagulation du Sang et les Sérum Anticoagulants, Ann. de l'Inst. Pasteur, 1901, 15, p. 129.

[4] Gengou, O.: Sur les Sensibilisatrices des Sérums Actifs contre les Substances Albuminoides, Ann. de l'Inst. Pasteur, 1902, 16, p. 735.

[5] Bauer, J., and St. Engel: Studien über das Fibrinogen, Biochem. Ztschr., 1912, 42, p. 399.

ment fixation showed individual and species-specificness, the precipitin test giving the same result with some overlapping. Ten years later Kato,[16] whose work has been accessible only in an abstract, found that the serum of a rabbit injected with a mammalian fibrinogen may react with it in the precipitin test and also with that of other species, but not with rabbit fibrinogen; further, that with fowl fibrinogen there may be only a slight reaction or none at all. Kato [6] also found that the serum of rabbits given injections with rabbit fibrinogen though not reacting with it may react with other fibrinogens. Davide [7] injected animals with fibrinogen and studied the effect of their serum on the living animal and on red blood cells, but as he did not make any observations on the precipitin reactions of fibrinogen or antifibrinogen serum, his work need not be considered at this time. Cornelia M. Downs [8] found the tissue fibrinogen in beef and swine "highly species-specific antigenically," but not limited by species as a coagulant. Without any knowledge of Kato's work, we began to study the precipitin reaction of fibrinogen and we have reported results [9] that in the main parallel his. The purpose of this article is to extend our report with additional results.

The various fibrinogens used in our experiments were prepared essentially as follows:[10] Four parts of the clear plasma obtained by thorough centrifugalization of oxalated blood are mixed with one part of saturated solution of ammonium sulphate; the precipitate is removed by centrifugalization or by straining through washed cheesecloth, depending on its state of division, and then washed with one or two changes of 20% saturated ammonium sulphate solution, and finally dissolved in a 2% solution of pure sodium chloride, and the solution filtered. A second precipitation is made by adding one part of saturated ammonium sulphate solution to five parts of the 2% sodium chloride solution; the precipitate is again separated as indicated, washed, and redissolved in 2% sodium chloride solution. At least four precipitations are made in this way in all cases. If there is any indication of a positive benzidin test in the last solution, further precipitations are made until the solution no longer reacts with benzidin.

Rabbits are immunized by intravenous injections at intervals of three days with increasing quantities of solutions of fibrinogen which may vary in strength. In most instances, the quantities have been 2, 4, 6, 8 and 10 cc. but good results have been obtained with three injections of larger quantities also. For example, a rabbit received intravenously 15 cc. of swine fibrinogen 1 to 166, three days later 20 cc., after three days again 20 cc., and on the fourth day thereafter, the

[6] Kato, K.: Serologische Studien über das Fibrinogen, Centralbl. f. Bakteriol., I, Ref., 1924, 75, p. 353. (Original report in Mitt. d. Med. Gesellsch. z. Tokyo, 1922, 36.)

[7] Davide, Hans: The Action of Antifibrinogen Serum on Red Corpuscles, Acta med. Scand., 1925, Suppl. 13.

[8] Downs, Cornelia M.: Antigenic Properties of Tissue Fibrinogen, J. Infect. Dis., 1925, 37, p. 49.

[9] Hektoen, Ludvig; and Welker, William H.: The Precipitin Reaction of Fibrinogen, J. Am. M. A., 1925, 85, p. 434.

[10] Howell, W. H.: The Coagulation of Blood, Harvey Lectures, 1916-1917, p. 272. McLean, J.: A Laboratory Method for the Preparation of Fibrinogen, Bull. Johns Hopkins Hosp., 1920, 31, p. 453.

ring test with undiluted serum gave a definite precipitate with fibrinogen solution 1 to 40,000. And two rabbits that received similar injections of human fibrinogen 1 to 250 gave serum with a titer of 32,000 four days after the last injection. As a rule, the serum has been fairly rich in precipitin on the fourth day after the last injection; in some cases additional injections may be given in the hope of increasing the precipitin content of the serum. The beef, dog, human and horse fibrinogens did not give any reaction for the corresponding hemoglobin or as a rule for blood euglobulin, pseudoglobulin or serum albumin in tests of the solutions with the corresponding precipitin serums; in the case of sheep and swine fibrinogens, such tests were not made. The facts in this respect in the case of chicken fibrinogen are discussed later.

The results of the precipitin tests by the layer or contact method, read after one hour at room temperature, show that the precipitin serums for fibrinogen may have considerable potency. The figures indicate only approximately the highest dilutions in 0.9% salt solution of the fibrinogen solutions in which definite reactions developed after contact for one hour with undiluted antiserums. It should be borne in mind that the tests could not be made with serums of the same age or with fibrinogens of the same age. On standing fibrinogen solutions tend to precipitate and consequently to lose in strength. Hence the figures in the table are not by any means as accurate as appearances indicate. In most cases the antiserums did not contain precipitin for the corresponding blood serum or serum proteins except possibly in traces; in one serum against beef fibrinogen and in one against swine fibrinogen, the specific serum precipitins in each reached a titer of 1:1,000; one serum against chicken fibrinogen reacted with chicken serum at 1:8,000, but after immunization with other preparations (Arthur G. Cole) of chicken fibrinogen, potent antifibrinogen serums were obtained that had only feeble action on chicken serum (table 2). It appears to be difficult, if not impossible, to separate fibrinogen completely from euglobulin in chicken plasma. In one series of tests, precipitin serums for beef, dog and human fibrinogens did not react with the corresponding hemoglobin or blood proteins (euglobulin, pseudoglobulin, serum albumin). Not all serums against fibrinogen have been without action on the corresponding blood proteins, however, but in no case has this action been marked.

Table 1 also shows that the mammalian fibrinogen precipitins are not species-specific in the usual sense, as in every instance there was action on heterologous fibrinogens. So far, no interaction has been noted between antiserum for mammalian fibrinogen and chicken fibrinogen or vice versa (table 2), but further observations are indicated in view of Kato's result and in view of our results with plasma

(table 3). The antiserum for chicken fibrinogen reacted freely with other fowl fibrinogens, as may be seen in table 2. Bird fibrinogens should be studied on a larger scale, and it would be of interest to study fish fibrinogens also. In conjunction with Arthur G. Cole,[11] a special

TABLE 1

PRECIPITIN REACTIONS OF DIVERSE FIBRINOGENS

Antigens		Antifibrinogen Precipitin Serums							Normal Rabbit Serum
		Beef	Chicken	Dog	Horse	Human	Sheep	Swine	
Fibrinogens..	Beef......	150000	0	400	50000+	3000+	3000+	3000+	0
	Chicken..	0	20000	0	0	0	0	0	0
	Dog......	80000	0	40000	1000	+	0	0	0
	Horse....	40000	0	1000	70000+	0	1000	2000+	0
	Human..	60000	0	500	500	16000	1600	6000+	0
	Sheep....	30000	0	0	500	0	24000	6000+	0
	Swine....	50000	0	0	16000	0	8000	60000+	0
Serums.......	Beef......	1000	0	0	0	0	0	0	0
	Chicken..	0	0	0	0	0	0	0	0
	Dog......	0	0	0	0	0	0	0	0
	Horse....	0	0	0	20	0	0	0	0
	Human..	0	0	0	0	0	0	0	0
	Sheep....	100	0	0	0	0	0	100	0
	Swine....	0	0	0	0	0	0	1000	0

The figures indicate approximately the highest dilutions of the fibrinogen solutions that developed precipitates at the plane of contact with undiluted antiserum after one hour at room temperature. The dilutions were made with 0.9% salt solution. 0 = no reaction.

TABLE 2

THE REACTIONS OF CHICKEN AND OTHER FOWL FIBRINOGENS

Antigens		Serum Against Chicken Fibrinogen	Serums Against Mammalian Fibrinogens (see tables 1 and 3)
Fibrinogens..	Chicken..........................	200000	0
	Duck............................	50000	0
	Goose...........................	25000	0
	Guinea hen......................	16666	0
	Turkey..........................	33332	0
	Mammalian......................	0	+
	(beef, dog, human, horse, rabbit, sheep, swine)		
Plasma.......	Chicken..........................	+	0
	Duck............................	+	0
	Goose...........................	+	0
	Guinea hen......................	+	0
	Turkey..........................	+	0
	Mammalian (see table 3)........	0 with exceptions	+
Serum........	Chicken..........................	100	0
	Duck............................	10	0
	Pigeon..........................	10	0
	Mammalian (see table 1)........	0	+ in some cases

+ = reaction, 0 = no reaction.

study is under way of the relation of fibrinogen to the other proteins of chicken blood.

The apparently different quantitative relations of the mammalian fibrinogens to the various antiserums suggest that there are differences as well as similarities in these fibrinogens. This is indicated also by the

[11] Hektoen, Ludvig; and Cole, Arthur G.: The Preparation and Precipitin Reactions of Egg Albumin and Blood Proteins of the Domestic Fowl, J. Infect. Dis., 1927, 40, p. 647.

results of absorption tests. Experiments with antiserum for swine fibrinogen, in which the antiserum was mixed in each case with an equal part of a solution of foreign fibrinogen from 1 : 200 to 1 : 800 in strength, and the eventual precipitate removed by centrifugalization, resulted in the removal of the corresponding precipitin, leaving the precipitin for swine fibrinogen in nearly its original strength or more or less reduced. It was found further that absorption with beef fibrinogen would remove the precipitin for beef and also for human fibrinogen and vice versa, and that absorption with sheep fibrinogen removed the precipitin for sheep and also for beef and human fibrinogens. On the other hand, treatment of serum against beef fibrinogen with equal parts of approximately 1 : 400 solutions of beef, horse, human, or swine

TABLE 3

Precipitin Reaction of Plasma with Antifibrinogen Serums

Plasma	Antifibrinogen Precipitin Serums							Normal Rabbit Serum
	Beef	Chicken	Dog	Horse	Human	Sheep	Swine	
Beef...	+	0 and +	+	+	+	..	+	0
Chicken...	..	+	+	0	0	..	0	0
Dog...	+	0	+	+	+	+	+	0
Goat...	+	0	+	+	+	+	+	0
Guinea-pig...	+	0	+	+	+	+	0	0
Horse...	+	0	+	+	+	+	+	0
Human...	+	0 and +	+	+	+	+	+	0
Rabbit...	+	0	+	Trace ?	Trace ?	Trace ?	Trace ?	0
Rat...	+	0	+	+	+	+	0	0
Sheep...	+	0	+	+	+	+	+	0
Swine...	+	0 and +	+	+	+	+	+	0

+ = reaction, 0 = no reaction.

fibrinogen removed all the precipitins except that horse fibrinogen did not take out all the precipitin for human fibrinogen. A serum against human fibrinogen mixed with the same amount of beef fibrinogen 1 to 200 lost all its precipitin for beef fibrinogen without much reduction in the precipitin for the human fibrinogen. Analysis by absorption should be carried further, but the indications are that mammalian fibrinogens include different antigens, some of which are common to at least several species.

As fibrinogen solutions and precipitin serums for fibrinogen stand in the icebox, their precipitin reactions after some weeks tend to become more narrowly species-specific; in other words, it appears as if certain precipitinogens and precipitins are lost after a time.

While antifibrinogen serum may not react with normal serum (except perhaps with the homologous as in the case of certain beef, swine and chicken fibrinogen precipitins), the results with mammalian

plasma in fully controlled tests are quite the opposite. Citrated plasma has been used, prepared by mixing one part of blood as drawn with two parts of 2% sodium citrate solution in 0.9% salt solution and centrifugating until the plasmatic fluid became perfectly clear. Besides the plasma from the mammals already enumerated, goat, guinea-pig, rabbit and rat plasma has been tested with the antifibrinogen serums used in this work. In most instances prompt reactions took place; dog, horse, human and swine plasma responding best with all the antiserums. Rabbit plasma, when fresh, appeared to react slightly with all the antifibrinogens, except chicken, but the results proved rather difficult to read on account of clotting. Guinea-pig and rat plasma did not react with old human or swine antifibrinogen, but did react with more recently prepared anti-

TABLE 4

REACTIONS OF SERUMS AGAINST BEEF AND HUMAN FIBRINOGEN WITH PLASMA

Plasma	Serum Against Beef Fibrinogen	Serum Against Human Fibrinogen
Beef...	+++++++	....
Chicken..	+	....
Dog...	+++++	++++++
Goat..	++	+
Guinea-pig....................................	++	0
Horse...	+++++++	+++++
Human...	++++++	++++++
Rabbit..	+	0
Rat...	+	0
Sheep...	+++	....
Swine...	++	+++++

In table 4 the + sign indicates the highest dilutions of citrated plasma (blood, one part, 2% sodium citrate in salt solution, 2 parts) that gave a definite precipitin reaction. The progressive dilutions in this case were made by adding an equal amount of salt solution to each previous mixture. In the tests of human plasma with serum against human fibrinogen, the amount of fibrinogen in the highest positive dilution of plasma was calculated to be 1 to 64,000; the antiserum in question reacted with pure human fibrinogen in an approximate dilution of 1 to 32,000. In other comparative tests similar results were obtained.

serums. The chicken antifibrinogen serum occasionally gave doubtful traces of reaction with beef, human and swine plasma. In the tests of plasma with various antifibrinogen serums, the positive reactions seemed to develop equally well whether the dilutions of the plasma were made with plain 0.9% NaCl solution or with 2% of sodium citrate in the salt solution. No effort has been made to determine the quantities of fibrinogen in the plasma of different animals in relation to the precipitin reactions, which varied notably as determined by the highest dilution of plasma giving a distinct precipitate by the contact method after one hour at the room temperature. In practically all cases the plasma gave positive reactions in the highest dilution in contact with its own (homologous) antiserum.

In addition to the reaction of rabbit plasma with antifibrinogen serum, it is of interest that in our experiments, and in this respect our

results deviate from Kato's, rabbit fibrinogen reacted with the serum of rabbits immunized with alien fibrinogen. This raises the question whether the rabbit can produce precipitin on injection of its own fibrinogen. As the rabbit can produce precipitin in response to rabbit lens protein and rabbit thyroglobulin, it is reasonable to expect that it might respond to rabbit fibrinogen also, and Kato found that in rabbits injected with rabbit fibrinogen the serum contained precipitin for other fibrinogens but not for that of the rabbit. We have immunized rabbits with rabbit fibrinogen (which is difficult to keep in solution) and obtained precipitin reaction with both rabbit fibrinogen and plasma, but with fibrinogen no reaction was evident in dilutions above 1 : 400 approximately. The subject of the antigenic action of rabbit fibrinogen in rabbits should receive more attention.

SUMMARY

The fibrinogens of beef, dog, horse, human, sheep and swine blood are precipitinogenic. These fibrinogens, to which may be added those of the goat, guinea-pig, rabbit and rat have antigenic elements or properties that are more or less common. Consequently, fibrinogen is not necessarily wholly different for each of these species, as seems to be the case with serum proteins and hemoglobin, but to varying extent the same, resembling in this respect casein, lens proteins and thyroglobulin, and the principle of specificness for species does not seem to hold fully in the precipitin reaction of mammalian fibrinogen, but the possibility that there may be species-specific elements in fibrinogen as prepared ordinarily is not excluded. While the mammalian fibrinogens just enumerated are not strictly species-specific, there appears to be also some relationship between them and chicken fibrinogen so far as indicated by the precipitin reactions that have been observed. This fact suggests that mammalian and bird fibrinogens are not wholly distinct and different. The fibrinogens of the chicken, duck, goose, guinea hen, pigeon and turkey appear to have in large measure common precipitinogenic properties. Taken together the results of the precipitin reactions of fibrinogen accord well with the fact that fibrinogen, a normal globulin of the circulating blood, is sufficiently uniform throughout the animal kingdom to give a characteristic fibrin gel with pig's thrombin (Howell[10]). Finally, it seems probable that as it is possible to obtain precipitin serum that is practically specific for blood fibrinogen, the precipitin test may prove to be of value in efforts to trace the origin of fibrinogen and its relation to other substances.

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